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Traditional Chinese medicine herbal extracts of *Cibotium barometz*, *Gentiana scabra*, *Dioscorea batatas*, *Cassia tora*, and *Taxillus chinensis* inhibit SARS-CoV replication

Chih-Chun Wen[1,2,§], Lie-Fen Shyur[2,§], Jia-Tsrong Jan[3,§], Po-Huang Liang[4], Chih-Jung Kuo[4,5], Palanisamy Arulselvan[2], Jin-Bin Wu[1], Sheng-Chu Kuo[1,*], Ning-Sun Yang[2,*]

[1] Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung 404, Taiwan
[2] Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan
[3] Genomics Research Center, Academia Sinica, Taipei 115, Taiwan
[4] Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan
[5] Department of Population Medicine and Diagnostic Science, Veterinary Medical Center, Cornell University, Ithaca, NY, 14853 USA
[§] *Contributed equally*

Abstract

Development of anti-severe acute respiratory syndrome associated coronavirus (SARS-CoV) agents is pivotal to prevent the reemergence of the life-threatening disease, SARS. In this study, more than 200 extracts from Chinese medicinal herbs were evaluated for anti-SARS-CoV activities using a cell-based assay that measured SARS-CoV-induced cytopathogenic effect (CPE) *in vitro* on Vero E6 cells. Six herbal extracts, one each from Gentianae Radix (龍膽 lóng dǎn; the dried rhizome of *Gentiana scabra*), Dioscoreae Rhizoma (山藥 shān yào; the tuber of *Dioscorea batatas*), Cassiae Semen (決明子 jué míng zǐ; the dried seed of *Cassia tora*) and Loranthi Ramus (桑寄生 sāng jì shēng; the dried stem, with leaf of *Taxillus chinensis*) (designated as GSH, DBM, CTH and TCH, respectively), and two from Rhizoma Cibotii (狗脊 gǒu jǐ; the dried rhizome of *Cibotium barometz*) (designated as CBE and CBM), were found to be potent inhibitors of SARS-CoV at concentrations between 25 and 200 μg/ml. The concentrations of the six extracts needed to inhibit 50% of Vero E6 cell proliferation (CC_{50}) and 50% of viral replication (EC_{50}) were determined. The resulting selective index values ($SI = CC_{50}/EC_{50}$) of the most effective extracts CBE, GSH, DBM, CTH and TCH were > 59.4, > 57.5, > 62.1, > 59.4, and > 92.9, respectively. Among these extracts, CBM and DBM also showed significant inhibition of SARS-CoV 3CL protease activity with IC_{50} values of 39 μg/ml and 44 μg/ml, respectively. Our findings suggest that these six herbal extracts may have potential as candidates for future development of anti-SARS therapeutics.

Abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; CPE, cytopathogenic effect; TCM, traditional Chinese medicine

Keywords: Severe acute respiratory syndrome (SARS); Traditional Chinese medicine (TCM); Cytopathogenic effect (CPE); SARS 3CL protease; *Cibotium barometz*

Correspondence to:
Dr. Ning-Sun Yang. Agricultural Biotechnology Research Center, Academia Sinica. No. 128, Academia Sinica Rd. Sec. 2, Nankang District, Taipei 11529, Taiwan. Tel: + 886-2-2787-2067. Fax: + 886-2-2651-1127. E-mail: nsyang@gate.sinica.edu.tw
Dr. Sheng-Chu Kuo. Graduate Institute of Pharmaceutical Chemistry, China Medical University. No.91, Hsueh-Shih Road,Taichung, Taiwan 40402, Taiwan. Tel: + 886-4-2205-3366 ext 5608. E-mail: sckuo@mail.cmu.edu.tw

Introduction

Severe acute respiratory syndrome (SARS) is a highly infectious, life-threatening disease caused by a novel coronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV) (Drosten et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003). In 2003, SARS spread quickly in over 25 countries and caused 8098 probable SARS cases and 774 SARS-related deaths. Although various candidate drugs have subsequently been reported to attenuate the disease (Cinatl et al., 2005; Hoever et al., 2005; Holmes, 2003), there are currently still no clinically approved or recommended antiviral drugs specific for SARS use. Currently, the most frequent treatment for SARS is antiviral and supportive treatment using a combination of ribavirin and corticosteroids (Peiris et al., 2003; So et al., 2003). However, ribavirin is only marginally effective against the SARS virus, and shows serious adverse side effects (Cinatl et al., 2003; Stroher et al., 2004). Since the SARS outbreak, considerable effort has been put into antiviral research to evaluate drug candidates for anti-SARS-CoV activity to prevent possible re-emergence of the disease (Wen et al., 2007; Wu et al., 2004). Notably, glycyrrhizin from licorice roots and a number of glycyrrhizin derivatives have been shown to possess demonstrable anti-SARS-CoV bioactivities (Hoever et al., 2005; Wu et al., 2004). And the non-steroidal anti-inflammatory drug, indomethacin, was also found to confer potent antiviral activity against SARS-CoV (Amici et al., 2006). A number of traditional herbal medicines have also been reported to possess antiviral activity against SARS-CoV (Chen et al., 2008; Li et al., 2005; Lin et al., 2005; Ryu et al., 2010).

The SARS-CoV genome encodes various key protein molecules that may serve as potential targets for chemotherapeutic inhibition of viral infection and replication (Lai, 2005; Stadler et al., 2003). These vital targets include: the spike protein (S), the SARS-CoV main protease (3CL protease), the NTPase/helicase, the RNA-dependent RNA polymerase, the membrane protein (M), the envelope protein (E), and the nucleocapsid phosphoprotein (N) and possibly other viral protein-mediated processes (De Clercq, 2004; Gallagher and Buchmeier, 2001; Groneberg et al., 2005; Holmes, 2003; Kliger et al., 2005; Lai, 2005; Stadler et al., 2003). Anti-SARS-CoV agents that can inhibit SARS-CoV replication may be involved in inhibition of one or more of the above protein targets including SARS-CoV 3CL protease. This important protease regulates the proteolytic processing of replicase polypeptides into functional proteins, playing an essential role in viral replication (Chen et al., 2002; Kuo et al., 2004). SARS-CoV 3CL protease is thus an attractive target for drug candidates against SARS.

In this study, we first investigated the effects of extracts of traditional Chinese medicinal (TCM) herbs on anti-SARS-CoV activity using a Vero E6 cell-based cytopathogenic effect (CPE) assay. The herbal extracts with anti-SARS-CoV activity shown by the CPE assay were subsequently evaluated for inhibition of SARS-CoV replication using ELISA. These bioactive extracts were then further investigated for inhibition of SARS-CoV 3CL protease activity. Our findings demonstrate that six previously unreported specific herbal extracts may have potential for use as drug targets for future development of anti-SARS therapeutics.

Materials and Methods

Preparation of extracts from traditional Chinese medicinal herbs

More than 50 traditional Chinese medicinal herbs were prepared and used as shown in Figure 1. Briefly, plant materials were ground into a powder, immersed in 10-fold volume of water in a sonicator for 1 hour, and filtered. Three-fold volume of 95% ethanol was further added to the filtrate left for 1 hour and centrifuged at 10000g for 20 minutes, at 4℃. The supernatant was rotor-evaporated and then freeze-dried to obtain the E fraction, and the 70% ethanol precipitated fraction was

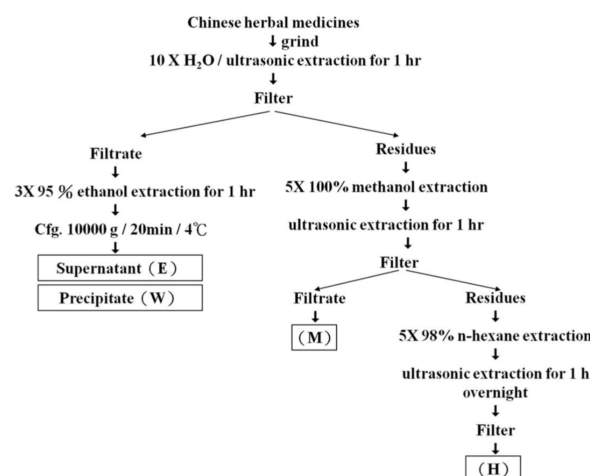

Figure 1. Schematic representation of different preparations of test herbal extracts. The E, W, M and H fractions from all tested plant materials were fractionated and dried as described in Materials and Methods.

freeze-dried to obtain the W fraction. The previous residue was mixed with 5-fold volume of methanol, left for 1 hour, and filtered again to obtain two fractions, the filtrate and the residue. The filtrate was rotor-evaporated, and further freeze-dried to obtain the M fraction. The residue was then mixed with 5-fold volume of n-hexane in a sonicator for 1 hour, kept overnight, filtered, rotor-evaporated, and further freeze-dried to obtain a filtrate (H). All plant materials were coded as the first letters of the name of the genus and species followed by the abbreviation for the preparative method. For example, the W, E, M and H extracts of *Cibotium barometz* were abbreviated as CBW, CBE, CBM and CBH, respectively.

Cytopathogenic effect of SARS-CoV on Vero E6 cells

Eight wells were prepared to test each extract: three wells contained virus-infection only with no test extract (as positive control for CPE); three wells contained virus-infection with extract treatment; and two wells contained extract treatment only, without viral infection. In brief, Vero E6 cells (2×10^4/well) were cultured in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) at 37℃ in an incubator with 5% CO_2 for one day. When cells reached 80-90% confluence, the culture medium was removed and replenished with 100 μL DMEM supplemented with 2% FBS. Test cell cultures at≧90% confluence were treated with or without tested extracts in a DMEM + 2% FBS medium. Two hours later, test cells in 50 μL of culture medium were incubated with SARS-CoV (Hong Kong strain) at a dose of 100 $TCID_{50}$ (50% tissue culture infectious doses) per well. The cytopathogenic morphology of cells was observed and evaluated at 72 hours post infection using inverted phase contrast microscopy.

Inhibition of SARS-CoV mediated CPE by the tested extracts was classified into three levels (+++, ++, +) as previously reported (Tan et al., 2004). Cell cultures in which less than 25% of Vero E6 cells showed cytopathogenic morphology in response to SARS-CoV after treatment with extracts were scored as +++. Cell cultures in which 25-50% and 50-70% cells showed cytopathogenic morphology were scored as ++ and +, respectively.

Cytotoxicity of test extracts on Vero E6 Cells

The assay protocol was as reported previously (Wen et al., 2007). Briefly, Vero E6 cells (2×10^4/well) were cultured in 96-well plates in DMEM supplemented with 10% FBS at 37℃ in a 5% CO_2 incubator. After incubation for one day during which cultured cells reached 90% confluence, the culture medium was replenished with 100 μL fresh DMEM medium containing 2% FBS and test extracts at varying concentrations, were placed into microwells and incubated for 3 days. The test culture medium was then replenished with 100 μL fresh culture medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 0.5 mg/mL per well for 4 hours. Optical density (OD) was then measured with a spectrophotometer at 570 nm. Survival of Vero E6 cells after treatment was calculated using the formula: viable cell number (%) = $[OD_{570\ (treated\ cells)}]/OD_{570\ (vehicle\ control\ cells)}] \times 100$. The CC_{50} value was taken to be the test compound concentration at which cell viability was reduced by 50%.

Inhibition of viral replication in SARS-CoV-infected Vero E6 cells

The inhibitory effects of test extracts on SARS-CoV replication were measured as previously described (Wen et al., 2007). Briefly, after test extracts had been added to Vero E6 cells and incubated for 3 days with SARS-CoV, the cells were gently rinsed with PBS three times and then fixed with 10% formalin for 5 minutes at room temperature. The 10% formalin was removed and the cells were fixed again in methanol/acetone (v/v, 1:1) solution for 5 minutes at room temperature. Cells were then blocked with 3% skim milk in PBS for 2 hours at room temperature, rinsed three times with PBS, and then incubated for 1 hour at 37℃ with 1:2,000 dilution of monoclonal antibody against the spike protein of SARS-CoV. All samples were then rinsed with three changes of PBS containing 0.05% Tween 20 (PBS-T buffer) followed by washing twice with fresh PBS at room temperature; and finally rinsed with 3% skim milk in PBS-T buffer. Cells were then incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG for 30 minutes at room temperature. After rinsing three times with PBS-T buffer, a substrate solution containing *o*-phenylenediamine dihydrochloride, citrate buffer (pH 5.0), and hydrogen peroxide was added to each well. Plates were covered and gently shaken at room temperature for 10 minutes in the dark. The reaction was stopped by addition of 2 N sulfuric acid, and absorbance was read immediately at 492 nm on an ELISA reader. The EC_{50} value for each test compound was calculated from a linear regression plot of compound concentration versus OD_{492}.

SARS-CoV 3CL protease inhibition assay

The gene encoding the SARS-CoV main protease was cloned from the whole viral genome by polymerase chain reaction (PCR) and primer insertion (forward primer 5′-GGTATTGAGGGTCGCAGTGGTTTTAGG-3′ and reverse primer 5′-AGAGGAGAGTTAGAGCCTTATTGGAAGGTAACACC-3′) into the pET32Xa/Lic vector as reported previously (Chen et al., 2002; Kuo et al., 2004). The recombinant 3CL protease plasmid was then transformed into *E. coli* JM109 competent cells that were streaked on a Luria–Bertani (LB) agar plate containing 100 μg/mL of ampicillin. The correct construct was subsequently transformed into *E. coli* BL21 host cells for expression of the His-tagged protein, which was then digested with FXa protease to remove the tag. The purified protein was confirmed by N-terminal sequencing and mass spectrometry analysis. The enzyme concentration used in all experiments was measured by the absorbance at 280 nm.

The kinetic measurements were performed in a solution containing 20 mM bis[(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (pH 7.0) at 25℃. Enhanced fluorescence resulting from cleavage of the fluorogenic substrate peptide (Dabcyl-KTSAVLQ-SGFRKME-Edans) of SARS 3CL-protease was monitored on a fluorescence plate reader (538 nm emission, 355 nm excitation). The initial velocities of the inhibiting activities on 50 nM SARS 3CL-protease using 6 μM fluorogenic substrate were plotted against the different inhibitor concentrations to obtain IC_{50} values using Equation 1 (Eq. 1), where $A[I]$ is the enzyme activity with inhibitor concentration $[I]$; and $A[0]$ is the enzyme activity without interference from an inhibitor:

$$A[I] = A[0] \times \{1 - [[I]/([I] + IC_{50})]\} \qquad \text{(Eq. 1)}$$

Results

Anti-SARS-CoV activity of test extracts as measured by cell-based cytopathogenic effect (CPE) assay

The anti-SARS-CoV activity of test extracts was first measured using a cell-based assay of the cytopathogenic effect on infected Vero E6 cells as described previously (Wen et al., 2007). Figure 2, panel A, shows the original morphology of the Vero E6 cells without treatment with test extracts (negative control), and panel B shows the cytopathic morphology of SARS-CoV-infected Vero E6 cells (positive control). Inhibition of SARS-CoV mediated CPE in Vero E6 cells was graded into three levels +, ++ and +++ (where + represented least inhibition, and +++ represented most inhibition) as shown in panels C, D, and E, respectively. Valinomycin (VAL), which has previously been reported to exhibit strong anti-SARS bioactivity *in vitro* (Wu et al., 2004), was employed as a reference control with high inhibition (level +++) (Table 1). Among all tested extracts, none of the W fractions from test plant materials conferred significant anti-SARS activity. For E, M and H fractions from all herbal extracts, only six extracts, CBE and CBM from Rhizoma Cibotii (狗脊 gǒu jǐ; the dried rhizome of *Cibotium barometz*), GSH from Gentianae Radix (龍膽 lóng dǎn; the dried rhizome of *Gentiana scabra*), DBM from Dioscoreae Rhizoma (山藥 shān yào; the tuber of *Dioscorea batatas*), CTH from Cassiae Semen (決明子 jué míng zǐ; the dried seed of *Cassia tora*), and TCH from Loranthi Ramus (桑寄生 sāng jì shēng; the dried stem, with leaf of *Taxillus chinensis*) (designated as GSH, DBM, CTH and TCH, respectively), and two from showed inhibitory activities (at levels + to +++) in the CPE assays at

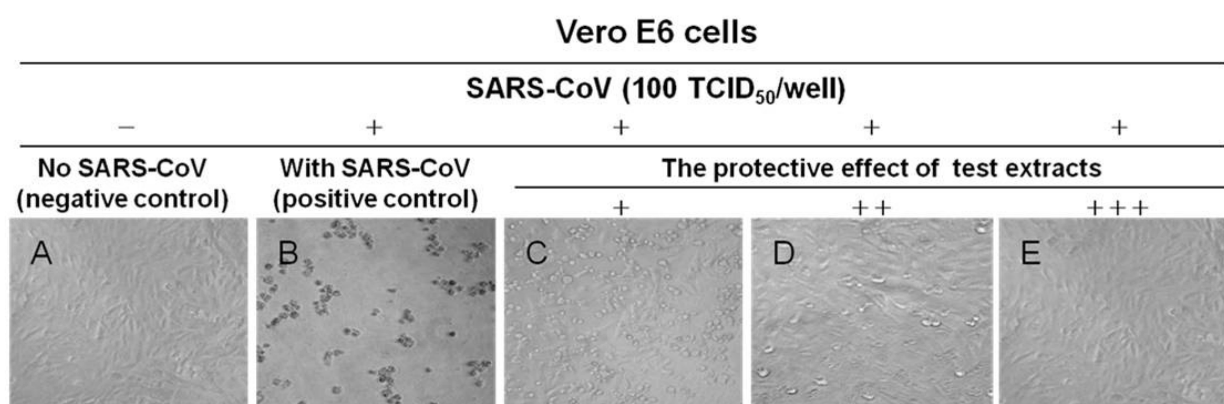

Figure 2. Inhibition of cytopathogenic effect (CPE) of SARS-CoV-infected Vero E6 cells by TCM phytoextracts. Representative cell-culture phenotypes or behavior of Vero E6 cells with or without infection with SARS-CoV are shown in A and B, respectively. C, D, and E are semiquantitative representations of the three levels of CPE inhibition (low +, moderate ++, and high +++), as revealed by phase contrast microscopy.

Table 1. Effect of Traditional Chinese Medicine extracts on cytopathogenic effect (CPE) of SARS-CoV on Vero E6 cells

Sample	Extract code	Concentration (μg/ml)				
		200	100	50	25	0
Rhizoma Cibotii (狗脊 gǒu jǐ)	CBE	+++	++	+	+	—
Rhizoma Cibotii (狗脊 gǒu jǐ)	CBM	+++	+	+	—	—
Gentianae Radix (龍膽 lóng dǎn)	GSH	+++	++	+	+	—
Dioscoreae Rhizoma (山藥 shān yào)	DBM	+++	++	+	+	—
Cassiae Semen (決明子 jué míng zǐ)	CTH	++	++	+	+	—
Loranthi Ramus (桑寄生 sāng jì shēng)	TCH	+++	+++	++	+	—
Valinomycin	VAL	N.T.	N.T.	N.T.	+++	—

[a] N.T., not tested

concentrations between 25 and 200 μg/ml (Table 1). To evaluate whether the vehicle solvent (0.2 - 0.4% DMSO) can result in possible cytotoxic or negative effects on Vero E6 cells, MTT assay and microscopic examinations were performed. Our results showed little or no cytotoxic effect was detectable in 0.2-0.4% DMSO-treated cells, as in good agreement with our previous findings (Wen et al., 2007).

Inhibition of SARS-CoV replication evaluated using ELISA.

To investigate whether the six herbal extracts that exhibited potent inhibitory activity could significantly inhibit viral replication, levels of spike protein in SARS-CoV infected Vero E6 cells with or without treatment with test extracts, were measured by ELISA. As seen in Figure 3A the six extracts showed anti-SARS-CoV replication activities at concentrations of 0.1 - 10 μg/ml as detected by ELISA. The concentration of each test extract required to inhibit 50% of viral replication (EC_{50}) was calculated and summarized in Table 2. In contrast to CBM which had EC_{50} values higher than 10 μg/ml, the EC_{50} values of CBE, GSH, DBM, CTH and TCH were determined at 8.42, 8.70, 8.06, 8.42 and, 5.39 μg/ml, respectively. Although these values are 2-3 fold higher than that of the reference compound valinomycin (VAL) (EC_{50} = 1.87 μg/ml), the considerably low EC_{50} values (< 10 μg/ml) of these extracts are very interesting, and may suggest that these test extracts apparently can significantly inhibit SARS-CoV replication with specificities.

Cytotoxic effects of test extracts on Vero E6 Cells.

Since it is possible that the anti-SARS-CoV activity of the test extracts may result from a direct inhibition on the growth of test Vero E6 cells, MTT assay was thus conducted to evaluate potential cytotoxic effect of test phytoextracts in concentrations ranging from 10 to 500 μg/ml on Vero E6 cells. As seen in Table 2 and Figure

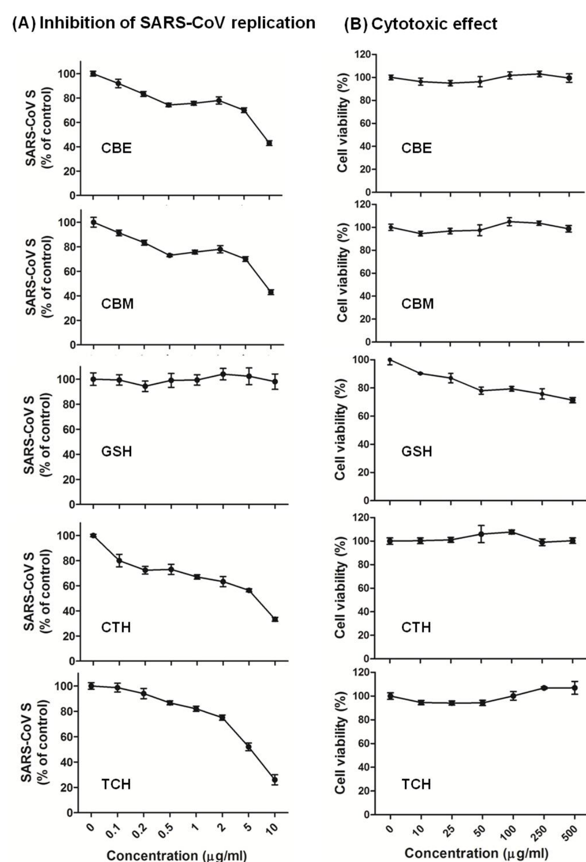

Figure 3. Inhibitory effect of test extracts on replication of SARS-CoV and on proliferation of Vero E6 cells. (A) Inhibition of SARS-CoV replication in response to treatment with specific extracts is measured by the level of SARS-CoV spike protein (SARS-CoV S) in test Vero E6 cell cultures using ELISA. % of Control = (OD_{492} of SARS-CoV infection – OD_{492} of mock infection [concn X]) / (OD_{492} of SARS-CoV infection – OD_{492} of Mock infection [concn 0]). (B) Cytotoxic effects of test extracts on Vero E6 cells were determined using MTT assay. Each data point represents the mean ± SD (n = 3). Cell viability (%) = (OD_{570} of treated cells / OD_{570} of vehicle cells) × 100.

Table 2. Effect of test extracts on Vero E6 cell proliferation and SARS-CoV replication

Sample	CC50 (μg/ml)[a]	EC50 (μg/ml)[b]	Selective Index[c]
CBE	>500	8.42	>59.4
CBM	>500	>10	N.C [d]
GSH	>500	8.70	>57.5
DBM	>500	8.06	>62.0
CTH	>500	8.43	>59.3
TCH	>500	5.39	>92.8
VAL [e]	75.01	1.81	41.4

[a] Determined as the cytotoxic concentration of test extracts that reduced cell viability to 50% of the control (i.e., cells with a treated equal volume of vehicle control). Each value was calculated with data obtained from triplicate samples.
[b] Determined as the effective concentration at which inhibition of viral replication was reduced to 50% of the untreated (control) cell cultures. Each value was calculated with data obtained from triplicate samples.
[c] Selective index was taken to be the ratio of CC_{50} to EC_{50} (CC_{50}/EC_{50})
[d] N.C., not calculable
[e] VAL., valinomycin

3B, the cytotoxic concentrations of individual extracts that reduced the cell viability to 50% of the untreated control (CC_{50}) were calculated and compared with each other. The CC_{50} values for all test extracts were detected to be higher than 500 µg/ml; interestingly, however, the positive control valinomycin had in fact a relatively low CC_{50} value (75.01 µg/ml). This result suggests that these extracts had little or no interference with the growth of Vero E6 cells, and they can be considered as generally biologically safe to the host cells. According to the results in Table 2, we hence suggest that it is quite unlikely that the inhibitory effects detected for the tested herbal extracts on viral replication of SARS-CoV were due to the inhibitory effect on cell growth or viability of host cells. In addition, the selective index (SI), the ratio of CC_{50} to EC_{50}, was also then calculated to evaluate the potency of anti-SARS-CoV activity of test extracts (Table 2). The SI values of CBE, CBM, GSH, DBM, CTH and TCH were determined at the value of > 59.4, not calculable, > 57.5, > 62.1, > 59.4, and > 92.9, respectively. These SI values, with the exception of CBM, are all higher than the value of the reference control valinomycin (SI = 41.4) as determined in parallel in this study and as we previously reported (Wu et al., 2004).

Inhibitory effects of test extracts on SARS-CoV 3CL protease activity.

The proteolytic cleavage of viral polyproteins at specific sites by 3CL protease plays an important role in SARS-CoV replication (Chen et al., 2002; Kuo et al., 2004). To understand whether the anti-SARS-CoV activity of these extracts can result from inhibition of SARS-CoV 3CL protease activity, all six test extracts were further evaluated in a 3CL protease inhibition assay. The IC_{50} values of extracts were measured using a quenched fluorescence energy transfer (FRET) method as previously described (Kuo et al., 2004). Niclosamide (NIC) has been reported to be an inhibitor of SARS-CoV 3CL protease activity and was used as a reference control: NIC had an IC_{50} value of 13 µg/ml in this study. As seen in Figure 4 and Table 3, among these extracts tested, only CBM and DBM conferred a considerable inhibition of SARS-CoV 3CL protease activity, with IC_{50} values of 39 µg/ml and 44 µg/ml, respectively. The IC_{50} values of the other test extracts were all higher than 50 µg/ml.

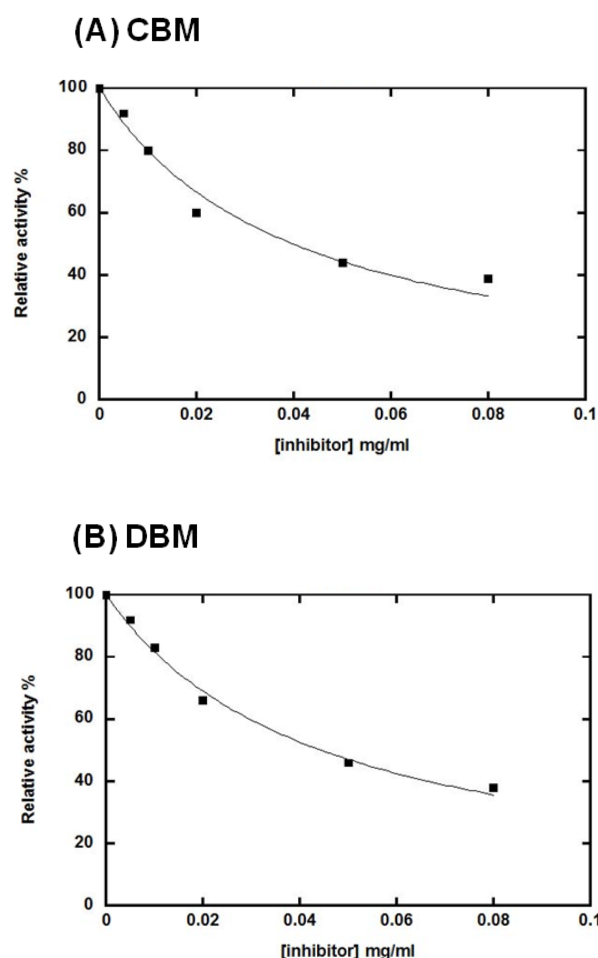

Figure 4. Inhibition of the enzymatic activity of SARS-CoV 3CL protease by CBM and DBM. The initial velocities of the inhibitory activities on 50 nM SARS 3CL-protease using 6 µM fluorogenic substrate were plotted against the different inhibitor concentrations (0 – 0.08 mg/ml) of CBM (A) and DBM (B) to obtain the IC_{50} values using Eq. 1, as described in Materials and Methods.

Table 3. IC_{50} values of test extracts on the enzymatic activities of SARS-CoV 3CL protease

Sample	IC_{50} (µg/ml)
CBE	>50
CBM	39 ± 3
GSH	>50
DBM	44 ± 2
CTH	>50
TCH	>50
NIC a	13. ± 0.7

[a] NIC, niclosamide

Conclusion

Since the outbreak of SARS in 2003, considerable effort has been put into research of the disease; however, to date, no drug has been approved for potential future clinical use in patients with SARS (Cleri et al., 2010). To assure adequate public safety and control of infection in the event of a re-emergence of this illness, effective anti-SARS-CoV agents may still be highly desirable or even necessary. Plant materials, especially for those that were previously used in traditional Chinese medicines, we believe are rich resources for development of the related therapeutic agents or drug candidates. In this study, we have employed a cell-based cytopathogenic effect (CPE) assay in SARS-CoV-infected Vero E6 cells for screening more than 200 extracts for candidate anti-SARS-CoV activity. Six herbal extracts from traditional Chinese medicinal herbs were found to exhibit significant levels (+ to +++) of anti-SARS-CoV activity at concentrations of 25 and 100 μg/ml (Table 1). As measured and compared by results from the CPE assays, this level of anti-SARS-CoV activity is better than that reported for glycyrrhizin (Cinatl et al., 2003), a compound with known anti-SARS-CoV activity, and extracts of other traditional herbs previously reported to confer anti-SARS-CoV activity (Lau et al., 2008; Li et al., 2005).

The CPE of viral infection may involve complex interactions of a number of molecular mechanisms between the SARS-CoV and test Vero E6 cells (Stadler et al., 2003). By quantification of the amount of spike protein present in SARS-CoV-infected Vero E6 cells (EC_{50}), we showed that the anti-SARS-CoV activity of these extracts may act specifically on the inhibition of viral replication (Table 2 and Figure 3A). In addition, a MTT assay was adopted to determine the CC_{50} of test extracts, and our data led us to suggest that the anti-viral activities we detected were apparently not due to the effects of cytotoxicity of test extracts on the host cells, as evidenced by the low EC_{50} of the extracts. The selective index (SI) value was calculated as the ratio of CC_{50} to EC_{50} as an indicator of the potency of test phytoextracts. In comparison to the positive control, valinomycin (VAL) (Wu et al., 2004), CBE, GSH, DBM, CTH and TCH showed potent activities against CPE, and they also exhibited marked inhibitory effects on SARS-CoV replication. The SI values (Table 2) for these five bioactive extracts are significantly higher even than the SI value of the positive control valinomycin (SI = 41.4). The SI values (all > 55) of these five extracts are also higher than two other traditional Chinese herbs previously reported to inhibit SARS-CoV infection; *Cinnamomi cortex* was shown to inhibit SARS-CoV infection at a SI value of 23.4, and *Toona sinensis* extract was shown to inhibit SARS-CoV replication with an SI value of (12 to 17) (Chen et al., 2008). Taken together, we hence suggest that the six TCM phytoextracts reported here can inhibit SARS-CoV replication with little or no cytotoxicity to Vero E6 cells, and are may thus serve as useful candidates for future development of anti-SARS therapeutics.

The SARS-CoV 3CL protease is involved in the viral maturation process by cleaving the virus-encoded polyproteins (Chen et al., 2002). Because of its pivotal role in the SARS-CoV life cycle, the 3CL protease is recognized as an important target for discovery of anti-SARS-CoV agents. Among the extracts tested for inhibition of SARS-CoV 3CL protease activity, the IC_{50} values of CBM (39 μg/ml) and DBM (44 μg/ml) suggest that these two extracts can exhibit significant inhibitory activity against 3CL protease. A number of studies have contributed to the identification of inhibitors of SARS-CoV 3CL protease [15, 28-29]. However, the IC_{50} values of CBM and DBM for inhibition of SARS-CoV 3CL protease are also similar or higher than extracts from other anti-SARS-CoV medicinal herbs reported to date, including *Isatis indigotica* (IC_{50} = 53.8 μg/ml) (Li et al., 2005), *Torreya nucifera* (IC_{50} = 100 μg/ml), tea extract (IC_{50} = 125 μg/ml) (Chen et al., 2005) and *Houttuynia cordata* (IC_{50} = 1000 μg/ml) (Lau et al., 2008). We therefore suggest that the herbal extracts of CBM and DBM may be useful drug candidates against SARS-CoV 3CL protease and may have potential for use as alternative herbal therapies against SARS. The other four herbal extracts we tested other than these two herbal extracts may inhibit SARS-CoV replication via different working mechanisms including blocking of viral binding, inhibition of SARS-CoV fusion with the host cells, inhibition on activity of other SARS-CoV proteases and inhibition of RNA transcription (Kliger et al., 2005).

Recently, phytochemicals or extracts derived from traditionally used medicinal plants or herbs have been contemplated or clinically evaluated as alternative or complementary treatments for various diseases. Previous reports showed that *Cibotium barometz* inhibited osteoclast formation and has antioxidative, tyrosinase inhibiting and antibacterial activities (Cuong et al., 2009; Lai et al., 2009). In this study, *Cibotium barometz* was found to possess good anti-SAR-CoV activity and effectively inhibit SARS 3CL

protease activity. The bioactive components responsible for these activities should therefore warrant further investigations. *Gentiana scabra* has previously been shown to confer a hepatoprotective effect (Jiang and Xue, 2005) and contains triterpenoids of secoiridoid and its glycosides (Chueh et al., 2001; Kakuda et al., 2001; Kim et al., 2009). Based on our previous study on anti-SARS-CoV phytocompounds (Wen et al., 2007), specific triterpenoids have been shown to inhibit SARS-CoV replication. We therefore suggest here that secoiridoid and its glycosides may contribute to the anti-SARS activity detected for *Gentiana scabra extract*, and secoiridoid and its glycosiedes may have potential for further evaluation as anti-SARS lead compounds. In our previous study (Su et al., 2011; Su et al., 2008), two specific polysaccharide-containing fractions from *Dioscorea batatas* tuber extracts significantly increased GM-CSF promoter activity in normal and inflamed skin, enhanced murine splenocyte proliferation *ex vivo* and improved regeneration of bone marrow cells *in vivo*. Other studies also revealed that the ethanolic extract from *Dioscorea batatas* can exert anti-inflammatory effect through the inhibition of NF-κB-mediated iNOS and COX-2 expressions (Jin et al., 2010). Interference of cyclooxygenase-2 (COX-2) expression may be correlated with anti-SARS-CoV and other antiviral activities (Amici et al., 2006; Kim et al., 2010). We therefore hypothesize that the anti-SARS-CoV activities we demonstrated here may result from inhibition of COX-2 activity. *Cassia tora* has been found to confer anti-hypertensive (Hyun et al., 2009), anti-hyperlipidemia (Cho et al., 2007), anti-bacterial (Hatano et al., 1999), and anti-fungal properties (Kim et al., 2004). Anthraquinones including emodin, physcion, and rhein may be the active phytocompounds that confer these activities (Kim et al., 2004; Wu and Yen, 2004). Because emodin has already been found to exhibit anti-SARS-CoV activity via inhibition of the viral entry by binding with the spike proteins and interfering with the SARS-CoV 3CL protease activity, we hypothesize that the active phytochemical components responsible for anti-SARS-CoV activity of CTH may be contributed by emodin or another anthraquinone(s) suggested or known to present in CTH. *Taxillus chinensis* was shown to inhibit fatty acid synthase (Wang et al., 2008; Wang et al., 2006). Based on the previous study (Yi et al., 2004), which showed luteolin and quercetin can interfere with the entry of the virus to its host cells, we therefore hypothesize that specific glycosylated flavonoid and quercetin may play an effective role in this activity. Whether these flavonoids can also contribute to the detected anti-SARS activity warrants future investigation.

In summary, in this study we showed that six phytoextracts from Rhizoma Cibotii (狗脊 gǒu jǐ), Gentianae Radix (龍膽 lóng dǎn), Dioscoreae Rhizoma (山藥 shān yào), Cassiae Semen (決明子 jué míng zǐ), and Loranthi Ramus (桑寄生 sāng jì shēng;) can confer effective anti-SARS-CoV activity via inhibition of SARS-CoV replication. The CBM and DBM extracts also inhibited the 3CL protease activity of SARS-CoV. These findings suggest that these phytoextracts studied as a TCM experience may be valued as a useful approach for future development of anti-SARS-CoV therapeutic agents.

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