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In vitro inhibition of coronavirus replications by the traditionally used medicinal herbal extracts, *Cimicifuga rhizoma*, *Meliae cortex*, *Coptidis rhizoma*, and *Phellodendron cortex*

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Abstract

Background: A search for new anti-coronaviral drugs to treat coronaviral infections was motivated by an outbreak of severe acute respiratory syndrome (SARS).

Objectives: In order to find drugs that treat coronavirus infections, including SARS, we screened traditional medicinal herbal extracts and evaluated their antiviral activities on coronavirus replication.

Study design: We employed a plaque assay to evaluate the effect of 22 medicinal herbal extracts on virus replication. We determined the 50% effective concentration (EC₅₀) of each extract that was necessary to inhibit the replication of mouse hepatitis virus A59 (MHV-A59); we also determined 50% cytotoxic concentrations (CC₅₀) for each extract. Northern and Western blot analyzes were performed to investigate antiviral activity in MHV-infected DBT cells, including virus entry, viral RNA and protein expression, and virus release. Coronavirus specific inhibition was also demonstrated using porcine epidemic diarrhea virus (PEDV).

Results: *Cimicifuga rhizoma*, *Meliae cortex*, *Coptidis rhizoma*, *Phellodendron cortex* and *Sophora subprostrata radix* decreased the MHV production and the intracellular viral RNA and protein expression with EC₅₀ values ranging from 2.0 to 27.5 µg/ml. These extracts also significantly decreased PEDV production and less dramatically decreased vesicular stomatitis virus (VSV) production in vitro.

Conclusions: The extracts selected strongly inhibited MHV replication and could be potential candidates for new anti-coronavirus drugs.

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Keywords: Coronavirus replication; Antiviral; Medicinal herbal extracts

1. Introduction

Coronaviruses cause acute and chronic respiratory, enteric, and central nervous system disease in many species of animals, including humans (Weiss and Navas-Martin, 2005). Among animal pathogens, PEDV, porcine transmissible gas-

troenteritis virus, bovine coronavirus, and avian infectious bronchitis virus are of veterinary importance. PEDV has more recently been identified as the causative agent of severe entero-pathogenic diarrhea in swine (Pensaert and Debouck, 1978).

Coronavirus is a family of enveloped, single-stranded, positive-strand RNA virus with a helical nucleocapsid. MHV has been extensively studied as a prototype of coronavirus and a model for human disease; it contains 31 kb genomic RNA that encodes seven to eight genes (Lee et al., 1991; Weiss and Navas-Martin, 2005). MHV genes are expressed through a

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genome-sized virus specific mRNA and six or seven species of virus-specific subgenomic mRNAs with a 3'-coterminal nested set structure (Lai et al., 1981; Leibowitz et al., 1981). The RNA genome is packaged together with nucleocapsid (N) protein and three envelope proteins: M (membrane), S (spike), and E (envelope).

The development of coronavirus specific therapy has been hampered until recently due to its relatively low burden on human disease; however, identification of the SARS coronavirus (SARS-CoV) as the cause of SARS in the spring of 2003 stimulated research in this field. Since then, several anti-SARS agents have been tested for coronavirus-specific therapy; however, an effective SARS antiviral therapy has not yet been established (Groneberg et al., 2005; Haagmans and Osterhaus, 2006; Stockman et al., 2006). Ribavirin, a synthetic nucleoside with a broad antiviral activity, is most frequently administered as a SARS-antiviral agent in combination with corticosteroids; however, this has little activity against SARS-CoV in vitro (Cinatl et al., 2003). SARS-CoV specific monoclonal antibodies, pegylated interferon- α , siRNA, and several protease inhibitors have also been tested against SARS-CoV (in reviews, Cinatl et al., 2005; Groneberg et al., 2005; Haagmans and Osterhaus, 2006). Glycyrrhizin, pyridine N-oxide derivatives, ATPase and helicase inhibitors, and 4-aminoquinoline chloroquine have also been found to inhibit SARS-CoV replication in vitro (Balzarini et al., 2006; Cinatl et al., 2003; Keyaerts et al., 2004; Tanner et al., 2005). Wu et al. (2004) tested more than 10,000 agents, including more than 1000 traditional Chinese herbs, and found 50 active compounds. Interestingly, they found that 10 of these compounds, including aescin, reserpine, and ginsenoside-Rb1, came from natural products that have been used clinically (Wu et al., 2004).

In an effort to search for a coronavirus specific therapy, we screened 22 traditionally used medicinal extracts for their ability to reduce the virus in MHV-A59-infected mouse DBT cells. Among these, Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, Phellodendron cortex and Sophora subprostrata radix exhibited anti-MHV activity which has not been previously reported. We have also demonstrated that these extracts inhibit PEDV replication, suggesting that they could contain candidate compounds for anti-coronaviral therapy.

2. Materials and methods

2.1. Viruses and cells

The plaque-cloned A59 strain of MHV (Lai et al., 1981) and mouse DBT cells (Hirano et al., 1974) were used for virus preparation and titration. Vero cell attenuated PEDV DR13 (Hofmann and Wyler, 1988; Kweon et al., 1999) and VSV, the member of the Rhabdoviridae family with a non-segmented single-strand, negative-sense RNA genome, were propagated and titrated in Vero cells (ATCC, CCL-81).

2.2. Preparation of medicinal herbal extracts

All plant materials were purchased at the University Oriental Drugstore, Iksan, Korea. Voucher specimens were deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Plant material (50 g) was extracted with methanol under ultrasonic conditions for 3 h, followed by paper filtration. The filtrates were evaporated in vacuo to yield methanol soluble extracts. Extracts were dissolved in DMSO.

2.3. Northern and Western blotting

Virus-specific RNA was extracted from virus-infected cells as described previously (Makino et al., 1984). Northern blot analysis was performed with a ^{32}P -labeled random-primed probe as described (Fosmire et al., 1992). Seven hours post-infection, DBT cells were lysed according to the method described previously (Makino et al., 1991). Lysates were separated by SDS-PAGE (10%), transferred to polyvinylidene fluoride (PVDF) membranes and incubated with monoclonal antibodies against MHV S or N proteins. Horseradish peroxidase-conjugated anti-mouse secondary antibodies and ECL (Enhanced Chemical Luminescence) were employed to visualize the respective MHV proteins. The relative intensities of the viral mRNAs and S or N proteins were measured using the Fujifilm Image Gauge V4.0 program (Fuji film Science lab 2001).

2.4. Cell cytotoxic assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the cytotoxic effects of extracts on DBT and Vero cells. DBT and Vero cells were grown in 96-well microplates and subsequently incubated with serial dilutions of each extract for 12 h at 37 °C. Cell viability was evaluated by replacing the culture medium with 100 μl of MTT in DMEM, incubating for 3 h, and measuring the absorbance with a plate reader at 570 nm. The CC_{50} of selected extracts was calculated.

3. Results

3.1. Screening for coronavirus-specific drugs

In an effort to search for drugs that treat coronavirus infections, we examined the effects of 22 traditionally used medicinal extracts (100 $\mu\text{g}/\text{ml}$) on DBT cells during and after they were infected with 2 or 20 multiplicity of infection (MOI) of MHV-A59. MHV production at 12 h post-infection was analyzed by plaque assay. Coptidis rhizoma completely abolished MHV production at both 2 and 20 MOI (Table 1, left, data not shown). In addition, Cimicifuga rhizoma, Meliae cortex, Phellodendron cortex and Sophora subprostrata radix significantly decreased MHV production in the range of a

Table 1
Effects of six medicinal herbal extracts on MHV-A59, PEDV, and VSV production

	Relative titer of released virus ^a		
	MHV-A59 ^b	PEDV ^b	VSV ^b
Virus only	100.0	100.0	100.0
DMSO	101.2 ± 13.6	100.7 ± 21.6	118.0 ± 31.0
Cimicifuga rhizoma	0.0044 ± 0.0029	4.7 ± 1.2	12.2 ± 3.6
Meliae cortex	0.0198 ± 0.0195	6.7 ± 0.4	20.5 ± 10.5
Coptidis rhizoma	<0.0000	5.1 ± 1.5	29.9 ± 24.4
Phellodendron cortex	0.0024 ± 0.0012	5.9 ± 0.4	40.0 ± 3.8
Moutan cortex radicis	19.5 ± 3.5	79.2 ± 0.9	64.5 ± 15.0
Sophora subprostrata radix	4.9 ± 2.2	7.8 ± 0.6	10.8 ± 7.2

^a Fold reduction relative to MHV-A59, PEDV, and VSV titers of 1.0×10^8 , 4.6×10^4 , and 2.3×10^8 PFU/ml, respectively, by treatment with 100 µg/ml of the medicinal herb extracts indicated. The results represent the mean ± S.D. from three independent experiments.

^b DBT cells were infected with MHV-A59 at 2 MOI and Vero cells were infected with PEDV at 0.5 MOI or VSV at 2 MOI. After 12 h post-infection, the titers of MHV, VSV and PEDV were determined by plaque assay.

20-fold to 4- \log_{10} reduction at 2 MOI, and an 8–300-fold reduction at 20 MOI (Table 1, left, data not shown). Moutan cortex radicis decreased MHV production by approximately fivefold and fourfold at 2 and 20 MOI, respectively.

An impaired intracellular MHV RNA expression was evident at 7 h post-infection using 100 µg/ml of extract; this roughly correlated with a decrease in MHV production (Fig. 1). Interestingly, MHV RNAs were still expressed in Coptidis rhizoma-treated cells even though the virus production was completely abolished (Table 1 and Fig. 1),

suggesting that Coptidis rhizoma might also inhibit the coronavirus protein expression and/or assembly and release. Furthermore, in Cimicifuga rhizoma-treated cells, MHV RNAs were almost undetectable even though the level of virus production was similar to that of Phellodendron cortex-treated cells (Table 1 and Fig. 1); this suggests that Cimicifuga rhizoma might drastically inhibit coronavirus RNA expression.

As shown in Table 1(middle), a decrease in PEDV production verified the coronavirus-specific inhibition by these five extracts, even though PEDV was less dramatically affected than MHV-A59. Levels of VSV were the least affected (Table 1, right), suggesting that these extracts exhibit coronavirus-specific activity.

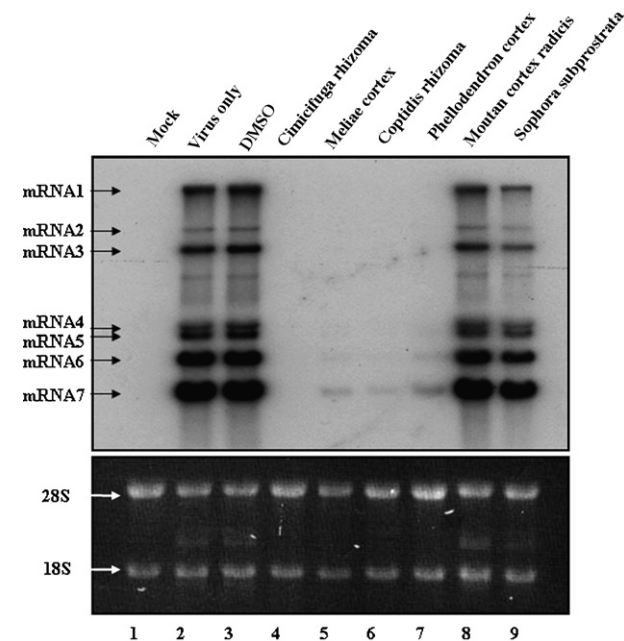


Fig. 1. Effects of medicinal herbal extracts on MHV-A59 RNA replication. MHV-A59 RNA expression was tested at 7 h post-infection in response to treatment with 100 µg/ml of extracts. Intracellular RNAs from mock-infected (lane 1), MHV-infected without treatment of extract (lane 2), MHV-infected with DMSO (lane 3), and MHV-infected and Cimicifuga rhizome-, Meliae cortex-, Coptidis rhizome-, Phellodendron cortex-, Moutan cortex radicis- and Sophora subprostrata radix-treated cells (lanes 4–9, respectively) were extracted at 7 h post-infection and analyzed by Northern blot analysis using a gene 7-specific probe. Seven species of mRNAs and 28S and 18S ribosomal RNAs are indicated.

3.2. Effects of selected extracts on MHV replication and cells

In order to determine the EC_{50} , varying concentrations of each extract (1–100 µg/ml) were applied to DBT cells from the adsorption period throughout the infection cycle. For all of the extracts tested, MHV production decreased in a dose-dependent manner; 50 µg/ml of Coptidis rhizoma completely abolished MHV production (Fig. 2). The EC_{50} of Cimicifuga rhizoma, Meliae cortex, Coptidis rhizoma, Phellodendron cortex, and Sophora subprostrata radix were 19.4 ± 7.0 , 13.0 ± 1.4 , 2.0 ± 0.5 , 10.4 ± 2.2 , and 27.5 ± 1.1 µg/ml, respectively (Table 2).

We also determined the CC_{50} of these extracts on DBT cells using an MTT-based cell viability assay. The CC_{50} was defined as the concentration of the extract necessary to reduce the cell viability to 50% of that of the control (cells without the addition of extracts); the results of this assay were in the range of 71.3 ± 7.2 – 334.5 ± 7.0 µg/ml (Table 2). The CC_{50} of Vero cells was much higher than 400 µg/ml for all of the extracts tested, with the exception of Cimicifuga rhizome which was 370 µg/ml (data not shown), indicating that DBT cells were very sensitive to the extracts tested. The selectivity indexes (CC_{50}/EC_{50}) for anti-MHV ranged from 11.1 to 34.9 (Table 2).

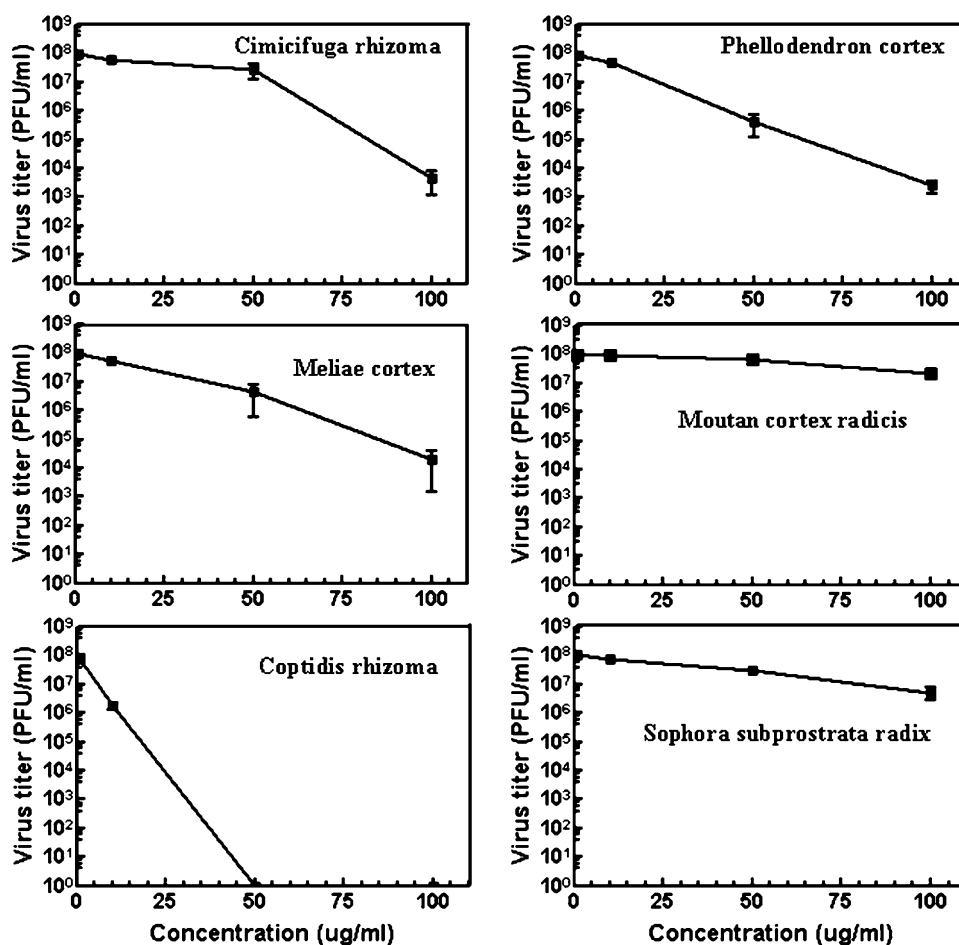


Fig. 2. Dose-dependent inhibition of MHV-A59 replication by Cimicifuga rhizoma, Meliae cortex, Coptidis rhizome, Phellodendron cortex, Moutan cortex radicis and Sophora subprostrata radix. Varying concentrations (1, 10, 50, 100 $\mu\text{g/ml}$) of six selected herbal extracts were applied to DBT cells, while they were simultaneously infected with MHV-A59 at 2 MOI, for 12 h at 37 °C. The virus titer was measured by plaque assay. Error bars represent the standard deviations from three independent experiments.

Table 2
Effects of six medicinal herbal extracts on MHV-A59

	EC ₅₀ ^a	CC ₅₀ ^b	SI ^c
Cimicifuga rhizoma	19.4 ± 7.0	239.0 ± 44.4	12.3
Meliae cortex	13.0 ± 1.4	334.3 ± 7.0	25.6
Coptidis rhizoma	2.0 ± 0.5	71.3 ± 7.2	34.9
Phellodendron cortex	10.4 ± 2.2	139.5 ± 81.3	13.4
Sophora subprostrata radix	27.5 ± 1.1	307.3 ± 6.6	11.1
Moutan cortex radicis	61.9 ± 6.1	598.7 ± 12.5	9.7

^a Determined as the concentration of extracts needed to inhibit the virus titer by 50% of the control value (cells without addition of extracts). Each value represents the mean ± S.D. from three independent experiments. The unit for EC₅₀ values shown in the table is $\mu\text{g/ml}$.

^b Determined as the concentration of extracts necessary to reduce the cell viability to 50% of the control (cells without addition of extracts). Each value represents mean ± S.D. from three independent experiments. The unit for CC₅₀ values shown in the table is $\mu\text{g/ml}$.

^c Selectivity index (CC₅₀/EC₅₀).

3.3. Inhibitory effects of the extracts on the replication cycle of MHV

Table 1 and Fig. 2 represent experiments in which the extract treatment and MHV infection were simultaneous. In order to investigate the effect of the extracts on different stages of the viral replication cycle we treated cells with 100 $\mu\text{g/ml}$ of extract at 2, 4, and 6 h post-infection and harvested MHV at 12 h post-infection. MHV production was decreased in these experiments (Fig. 3), although to a lesser extent than the reductions we observed when the addition of the extract and infection were simultaneous (Table 1 and Fig. 2). The antiviral activity of the extracts diminished as viral replication proceeded; hence, MHV production correlated with the time of extract treatment. These results were probably dependent upon the replication stage of the virus and the length of time it was exposed to the extracts (Fig. 3). Treatment with the extracts Cimicifuga rhizoma, Meliae cortex, Coptidis rhizome, Phellodendron cortex, Moutan cortex radicis, and Sophora subprostrata radix, at 6 h post-infection decreased

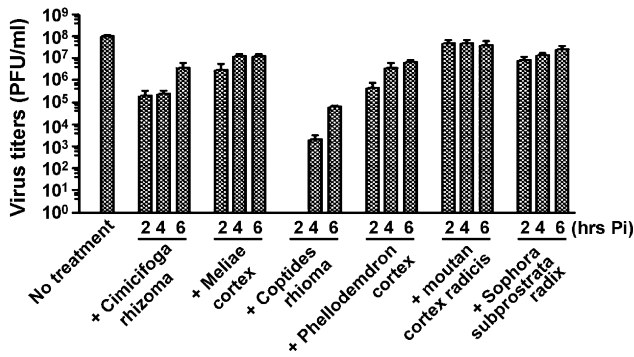


Fig. 3. Time-dependent inhibition of MHV-A59 replication by medicinal herbal extracts. The six selected herbal extracts, *Cimicifuga rhizoma*, *Meliae cortex*, *Coptidis rhizoma*, *Phellodendron cortex*, *Moutan cortex radices* and *Sophora subprostrata radix*, were applied to MHV-A59 infected DBT cells at 2, 4, and 6 h post-infection at 37 °C and were incubated until 12 h post-infection. The virus titer was measured by plaque assay. Error bars represent the standard deviations from three independent experiments. Pi indicates post-infection.

MHV production by 26-, 8-, 1612-, 15-, 2-, and 4-fold, respectively (Fig. 3).

In order to understand the mode of antiviral activity on viral entry and/or viral RNA and protein expression during MHV replication, EC₉₀ values were also determined (Fig. 2). The EC₉₀ of *Cimicifuga rhizoma*, *Meliae cortex*, *Coptidis rhizoma*, *Phellodendron cortex*, and *Sophora subprostrata radix* were 55.6 ± 4.2, 37.9 ± 8.8, 5.8 ± 0.6, 23.4 ± 1.2, 82.2 ± 8.2 µg/ml, respectively. We could not determine the EC₉₀ of *Moutan cortex radices* due to a low anti-MHV activity (Figs. 1 and 2, and Table 2). As expected, 10% of the MHV was produced when DBT cells were treated at EC₉₀ (data not shown). In order to analyze the inhibition on viral entry, one set of DBT cells was infected with 2 MOI of MHV-A59 in the presence of extracts (EC₉₀) from the adsorption period throughout the infection cycle. A second set of DBT cells were pre-adsorbed with MHV-A59 for 1 h at 4 °C without extracts, and un-adsorbed viruses were washed out. MHV-pre-adsorbed DBT cells were temperature-shifted to 37 °C at which temperature the viruses would be endocytosed, and they were further incubated with extracts at EC₉₀ until completion of the experiment. We expected to discern whether the extracts interfered with virus replication at the entry point, or during the subsequent transcription or translation processes by comparing the RNA and protein expression in these two sets of MHV-infected and extract-treated DBT cells. If MHV-pre-adsorbed DBT cells expressed more or the same amount of viral RNA and protein as those of the MHV-infected and simultaneously extract-treated cells, it would indicate that the extract interfered with viral entry. In this experiment, however, all of the MHV-pre-adsorbed cells expressed less viral RNA and protein than the other set of cells (Fig. 4), indicating that viral entry was not the main target of the extracts tested.

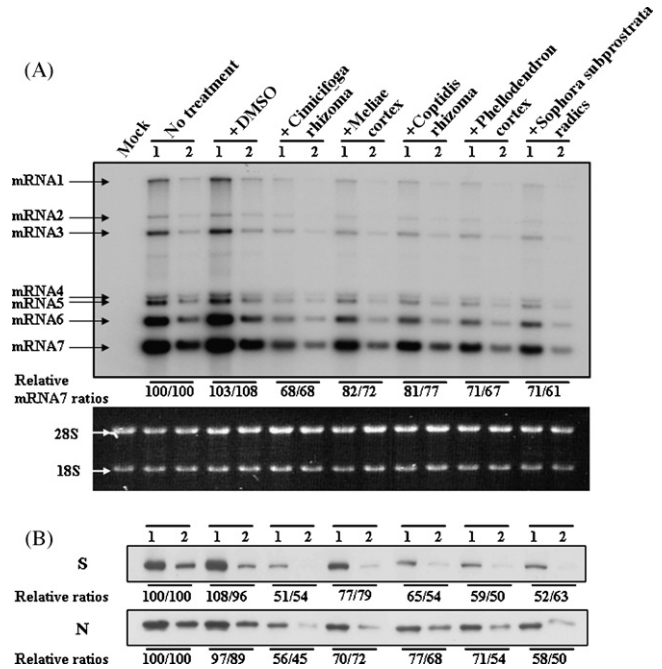


Fig. 4. Inhibitory effects of the extracts on the replication cycle of MHV at EC₉₀. (A) Virus-specific RNA expression in response to treatment with extracts at EC₉₀. Intracellular RNA was extracted at 7 h post-infection and analyzed by Northern blot analysis using a gene 7-specific probe. Lane 1 represents MHV mRNAs from simultaneously infected and extract-treated DBT cells. Lane 2 represents MHV mRNAs from MHV-pre-adsorbed, then temperature-shifted cells. The relative expression ratios of mRNA 7, which encodes N protein, from the simultaneously infected and extract-treated DBT cells (lane 1) and the MHV-pre-adsorbed, then temperature-shifted DBT cells (lane 2) were analyzed by densitometry (Fuji film Science lab 2001, Image Gauge Version 4.0). (B) Western blot analysis to detect MHV-specific N and S proteins in response to treatment with extracts at EC₉₀. Lysates at 7 h post-infection were subjected to 10% SDS-PAGE, transferred to PVDF membranes and incubated with monoclonal antibodies against MHV S or N proteins. The relative expression ratios of the respective S and N proteins from the simultaneously infected and extract-treated DBT cells (lane 1) and the MHV-pre-adsorbed, then temperature-shifted DBT cells (lane 2) were analyzed by densitometry (Fuji film Science lab 2001 Image Gauge Version 4.0).

4. Discussion

Numerous attempts have been made to identify treatments for coronavirus infections since the SARS outbreak (in reviews, Groneberg et al., 2005; Haagmans and Osterhaus, 2006; Stockman et al., 2006). Among them, several components from traditional Chinese medicine (TCM) and many small molecules of herbal origin have been shown to have some anti-SARS-CoV activities in vitro; this partly explains the beneficial effects of TCM in SARS patients (Cinatl et al., 2005; Wu et al., 2004).

Throughout the life cycle of coronavirus there are several potential targets for antiviral agents to interfere with during viral entry; these include the binding of the S protein to receptors on the target cell, and virus assembly and release through different replication steps. In the present study, we have demonstrated that viral entry was not the main target;

however, viral RNA synthesis was significantly decreased (Figs. 1 and 3A), suggesting that the underlying antiviral mechanism might be the inhibition of RNA-dependent RNA polymerase or proteases that are crucial for coronavirus RNA replication. Furthermore, RNA synthesis and N and S expression was inhibited more drastically in *Cimicifuga rhizoma*-, *Phellodendron cortex*-, and *Sophora subprostrata radix*-treated cells than *Melaleuca cortex*- and *Coptidis rhizome*-treated cells, indicating that *Melaleuca cortex* and *Coptidis rhizome* might also partly affect virus assembly or release.

It had been recently reported that curcumin obtained from *Curcuma longa* extract inhibits MHV-A59 replication by inhibiting cyclooxygenase activity, the prostaglandin H₂ synthase that converts arachidonic acid into prostaglandin (Raaben et al., 2007). We could not exclude the possibility that the extracts tested in our study also inhibited cellular mechanisms that are used by viruses during replication. This would be interesting to test in the future. Nonetheless, the decrease in MHV that we observed in response to extract treatment was very strong compared to that of VSV, suggesting that the extracts are specific MHV inhibitors.

Cimicifuga rhizome, which contains ferulic and isoferulic acids as its main active components, has anti-inflammatory activity (Sakai et al., 1999). *Melaleuca cortex*, containing toosendanin as one of its main components, has been used to treat ascariasis, oxyuriasis, scabies, tinea, and dermatosis (Lee et al., 2007). *Coptidis rhizoma* contains berberine and has anti-inflammatory effects that alleviate common dermatological disorders (Enk et al., 2007). *Phellodendron cortex* contains protoberberine alkaloids and has been used to treat meningitis, bacillary dysentery, pneumonia, tuberculosis and liver cirrhosis (Li et al., 2006). *Sophora subprostrata radix* contains matrine, oxymatrine, sophoranone, and sophocarpine and has been used as an antedote, an anodyne, and an anticancer agent (Kajimoto et al., 2002).

Cimicifuga rhizoma, *Melaleuca cortex*, *Coptidis rhizoma*, *Phellodendron cortex*, and *Sophora subprostrata radix* are good candidates to be anti-coronaviral agents for the treatment of coronaviral infections in both humans and animals.

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