Original article

Medicinal herbal extracts of *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* and *Torilis fructus* inhibit coronavirus replication *in vitro*

*Hye-Young Kim[1], Eun-Young Eo[1], Hyun Park[2,3], Youn-Chul Kim[2,4], Sun Park[1], Ho-Joon Shin[1], Kyongmin Kim[1,2]**

[1]Department of Microbiology, Ajou University School of Medicine, Suwon, South Korea
[2]Zoonosis Research Center, Wonkwang University, Iksan, Chonbuk, South Korea
[3]Department of Infection Biology, School of Medicine, Wonkwang University, Iksan, Chonbuk, South Korea
[4]College of Pharmacy, Wonkwang University, Iksan, Chonbuk, South Korea

*Corresponding author e-mail: kimkm@ajou.ac.kr

Background: *Cimicifuga rhizome*, *Meliae cortex*, *Coptidis rhizome* and *Phellodendron cortex* have been previously shown to exhibit anti-coronavirus activity. Here, an additional 19 traditional medicinal herbal extracts were evaluated for antiviral activities *in vitro*.

Methods: A plaque assay was used to evaluate the effects of 19 extracts, and the concentration of extract required to inhibit 50% of the replication (EC_{50}) of mouse hepatitis virus (MHV) A59 strain (MHV-A59) was determined. The 50% cytotoxic concentration (CC_{50}) of each extract was also determined. Northern and western blot analyses were conducted to evaluate antiviral activity on viral entry, viral RNA and protein expression, and release in MHV-infected DBT cells.

Results: *Sophorae radix*, *Acanthopanacis cortex* and *Torilis fructus* reduced intracellular viral RNA levels with comparable reductions in viral proteins and MHV-A59 production. The extracts also reduced the replication of the John Howard Mueller strain of MHV, porcine epidemic diarrhoea virus and vesicular stomatitis virus *in vitro*. *Sanguisorbae radix* reduced coronavirus production, partly as a result of decreased protein synthesis, but without a significant reduction in intracellular viral RNA levels. The EC_{50} values of the four extracts ranged from 0.8 to 3.7 μg/ml, whereas the CC_{50} values ranged from 156.5 to 556.8 μg/ml. *Acanthopanacis cortex* and *Torilis fructus* might exert their antiviral activities in MHV-A59-infected cells by inducing cyclooxygenase-2 expression via the activation of extracellular signal-related kinase (ERK) and p38 or ERK alone, respectively.

Conclusions: *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* and *Torilis fructus* might be considered as promising novel anti-coronavirus drug candidates.

Introduction

Coronaviruses (CoV) infect a broad range of mammals (including humans) and birds [1]. Diseases associated with various CoV include respiratory disease, gastroenteritis, hepatitis, neurological diseases and a syndrome similar to multiple sclerosis in humans, as well as a number of other illnesses [1]. Among animal pathogens, porcine epidemic diarrhoea virus (PEDV), porcine transmissible gastroenteritis virus, bovine CoV and avian infectious bronchitis virus are all of veterinary importance. PEDV has more recently been identified as the causative agent of severe enteropathogenic diarrhoea in swine [2].

The CoV are a family of enveloped, single-stranded, positive-stranded RNA viruses with genomes of 27–32 kb, with a helical nucleocapsid (N). Mouse hepatitis virus (MHV) has been studied extensively as a prototype CoV and also as a model for human disease; it harbours 31 kb of genomic RNA, which encodes 7 or 8 genes [1,3]. MHV genes are expressed via a genome-sized virus-specific messenger RNA (mRNA) and six or seven species of virus-specific subgenomic mRNAs with a 3′-coterminal nested set structure [4,5]. The RNA genome is packaged together with the N protein and three envelope proteins: M (membrane), S (spike) and E (envelope).

Up until recently, the development of CoV-specific therapies has been limited by the relatively low burden of disease these viruses inflict on humans;

however, the emergence of severe acute respiratory syndrome CoV (SARS-CoV) as the causative agent of SARS in the spring of 2003 stimulated a new surge of research into developing therapies against this group of viruses. Several anti-SARS agents have been tested for CoV-specific therapy; however, an effective SARS antiviral therapy has yet to be established [6–8]. Ribavirin, a synthetic nucleoside with broad antiviral activity, is administered most frequently in combination with corticosteroids; however, this combination therapy showed minimal activity against SARS-CoV *in vitro* [9]. SARS-CoV-specific monoclonal antibodies, pegylated interferon-α, small interfering RNA and several protease inhibitors have also been tested against SARS-CoV [6,7,10]. Glycyrrhizin, pyridine N-oxide derivatives, niclosamide (an antihelminthic drug), ATPase and helicase inhibitors, and 4-aminoquinoline chloroquine (malarial drug) have also been demonstrated to inhibit the replication of SARS-CoV *in vitro* [9,11–14]. Wu *et al*. [14] tested >10,000 agents, including >1,000 traditional Chinese herbs, and identified 50 active compounds. Of these compounds, 10 (including aescin, reserpine and ginsenoside-Rb) have been tested clinically; however, the mechanism underlying the action of these compounds has not been clearly established. Previously, we have shown that *Cimicifuga rhizoma*, *Meliae cortex*, *Coptidis rhizoma*, *Phellodendron cortex* and *Sophora subprostrata radix* have anti-MHV activity against A59 strain (MHV-A59)-infected mouse DBT cells [15]. We have also demonstrated that these extracts inhibit PEDV replication, suggesting that they might harbour candidate compounds for anti-CoV therapy [15].

In this study, we screened 19 additional traditional medicinal extracts, excluding the 22 extracts previously tested, for their ability to inhibit CoV replication. Additionally, we examined the mechanisms of action of these extracts. Among the extracts, *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix*, *Punicae cortex* and *Torilis fructus* showed anti-CoV activity, although such an effect has never been reported previously. Furthermore, in order to elucidate the antiviral effects of these extracts on MHV-A59 in greater detail, we evaluated their effects on the replication cycle of MHV-A59, including effects on viral RNA synthesis, protein expression and virus production. We also examined whether the inhibition of MHV-A59 replication correlated with the activation of extracellular signal-related kinase (ERK), p38 or c-Jun N-terminal kinase (JNK), and whether induced cyclooxygenase (COX)-2 expression was affected by the extract treatment of virus-infected cells. ERK, p38 or JNK activation and the induction of COX-2 expression were also evaluated in non-virally-infected cells. Our results indicated that ERK and p38 activation or ERK activation alone and the ensuing induction of COX-2 expression might be involved in the inhibition of MHV-A59 replication by these extracts. Collectively, our results indicate that the extracts reduced virus production via the inhibition of replication and/or by exerting effects on cellular signal transduction pathways.

Methods

Cells and viruses

DBT, an astrocytoma cell line, was maintained as previously described [16]. Vero cells, an African green monkey kidney cell line (CCL-81; ATTC, Manasas, VA, USA), were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. The plaque-cloned MHV-A59 and the John Howard Mueller strain of MHV (MHV-JHM) were propagated and titrated in DBT cells, as described previously [5]. Vero-cell-attenuated PEDV DR13 [17,18] and vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family possessing a non-segmented single-stranded negative-sense RNA genome, were propagated and titrated into Vero cells.

Preparation of medicinal herbal extracts

All plant materials were purchased from the Wonkwang University Oriental Drugstore (Iksan, Chonbuk, Korea). Voucher specimens were deposited at the Herbarium of the College of Pharmacy, Wonkwang University. 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. The extracts (Table 1 and Additional file 1) were dissolved in dimethyl sulfoxide (DMSO).

Northern and western blot analyses

Virus-specific cytoplasmic RNA was extracted from virus-infected cells at 4 or 5.5 h post-infection, as previously described [19]. Northern blot analysis was performed with a ^{32}P-labelled random primed MHV-specific probe, as previously described [20]. DBT cell lysates (0.1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride were prepared at 7 h post-infection and their protein concentrations were estimated using Bradford assays. Equal quantities of cell lysates were separated via SDS 10% PAGE, transferred to polyvinylidene fluoride membranes and incubated with monoclonal antibodies against MHV N, S or M proteins, followed by a secondary antibody coupled to horseradish peroxidase (1:2,000 dilution; DAKO, Glostup, Denmark). The blots were then developed with an enhanced chemiluminescence agent (ECL™ Western Blotting Detection Reagent;

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

Sample	Relative titre of released virus[a]			
	MHV-A59[b]	MHV-JHM[b]	PEDV[b]	VSV[b]
Virus only	100.0	100.0	100.0	100.0
DMSO	102.4 ±18.5	91.1 ±7.8	105.8 ±16.7	98.7 ±21.0
Sophorae radix	0.0054 ±0.0033	0.0220 ±0.0027	0.7425 ±0.2	15.8 ±4.6
Pulsatillae radix	21.8 ±6.1	31.8 ±5.9	68.7 ±2.3	48.9 ±12.2
Acanthopanacis cortex	0.0033 ±0.0003	0.0486 ±0.016	0.1302 ±0.016	10.8 ±2.6
Sanguisorbae radix	3.2 ±1.2	0.2193 ±0.048	3.5 ±0.6	41.2 ±12.1
Punicae cortex	17.3 ±3.3	3.9 ±0.6	4.1 ±0.9	39.5 ±12.3
Torilis fructus	<0.0000	<0.0000	<0.0000	2.8 ±0.6
Ribavirin	16.0 ±4.6	0.3131 ±0.06	ND	10.2 ±0.9

The results represent the mean ±SD from three independent experiments. [a]Fold reduction relative to mouse hepatitis virus (MHV) strains A59 (MHV-A59) and John Howard Mueller (MHV-JHM), porcine epidemic diarrhoea virus (PEDV) and vesicular stomatitis virus (VSV) titres of 6×10^7, 6.6×10^6, 5.8×10^4 and 2.8×10^8 plaque-forming units per ml, respectively, by treatment with 50 μg/ml of the indicated medicinal herb extracts or ribavirin. [b]DBT cells were infected with MHV-A59 and MHV-JHM at a multiplicity of infection (MOI) of 2 and Vero cells were infected with PEDV at 0.5 MOI or VSV at 2 MOI. After 12 h post-infection, the titres of MHV-A59, MHV-JHM, VSV and PEDV were determined using plaque assays. DMSO, dimethyl sulfoxide; ND, not determined.

Amersham Pharmacia Biosciences, Piscataway, NJ, USA). The relative intensities of the viral mRNAs and N proteins were measured using the FujiFilm Image Gauge version 4.0 programme (Fuji Film, Tokyo, Japan).

Cell cytotoxicity assays

The cytotoxic effects of the extracts on DBT and Vero cells were assessed using a colorimetric assay containing 3-(4,5-dimethylthiozol-2-yl)-2,5-dipheryltetrazolium bromide (MTT; Sigma, St Louis, MO, USA) as previously described [15]. The 50% cytotoxic concentrations (CC_{50}) of the extracts were also calculated.

RT-PCR and quantitative real-time RT-PCR

To determine the levels of COX-2 mRNA in DBT cells, reverse transcriptase (RT)-PCR was performed. In brief, total RNAs were extracted by RNA Stat 60 (Tel-Test, Inc., Friendswood, TX, USA) in accordance with the manufacturer's instructions. Each 3 μg of RNA was reverse transcribed with oligo-dT and PCR amplified. COX-2 mRNA was amplified using sense (5′-GAAATGGCTGCAGAATTG-3′) and antisense (5′-TCATCTAGTCTGGAGTGG-3′) primers. β-actin mRNA, which was used as an internal control, was amplified with sense (5′-CAGGTCATCACC ATTGGCAATGAG-3′) and antisense (5′-CAGCACT GTGTTGGCGTACAGGTC-3′) primers. The inducible nitric oxide synthase mRNA was amplified with sense (5′-CCTCCTCCACCCTACCAAGT-3′) and antisense (5′-CACCCAAAGTGCTTCAGTCA-3′) primers. Amplified complementary DNAs were electrophoretically analysed on 1% agarose gels. Quantitative real-time RT-PCR was also performed to quantify COX-2, interleukin (IL)-6 and tumour necrosis factor (TNF)-α mRNA expression levels. Primers for real-time RT-PCR are listed in Additional file 2.

Direct effects of extracts on virion and virion RNAs

MHV-A59 viruses were treated for 1 h with herbal extracts or DMSO at 37°C. DBT cells were infected with extract-treated viruses. Virion RNA was prepared from extract- or DMSO-treated viruses and transfected into new DBT cells using Lipofectin (Invitrogen, Gaithersburg, MD, USA). After 16 h of incubation at 37°C, the supernatants were analysed by a plaque assay. To exclude the possibility of RNase contamination of the extracts, MHV-A59 virion RNA was prepared, incubated with the extracts for 1 h and then analysed by agarose gel electrophoresis.

Statistical analyses

The data were analysed for statistical significance and expressed as mean values ±SD. The mean values were compared using Student's *t*-test. *P*-values of <0.05 were considered as statistically significant.

Results

Screening for CoV-specific drugs

We previously reported that the traditional medicinal herbal extracts, *Cimicifuga rhizome*, *Meliae cortex*, *Coptidis rhizome* and *Phellodendron cortex* were capable of inhibiting CoV production via reductions in viral RNA synthesis and viral protein expression [15]. In this study, the effects of an additional 19 medicinal herbal extracts (50 μg/ml) on DBT cells were evaluated during and after infection with a multiplicity of infection (MOI) of 2 or 20 of MHV-A59. MHV-A59 production at 12 h post-infection was analysed via a plaque assay. *Torilis fructus* inhibited MHV-A59 production completely at 2 MOI (Table 1) and resulted in a 1,022-fold reduction at 20 MOI (H-YK, data not shown). *Sophorae radix* and *Acanthopanacis cortex* significantly reduced MHV-A59 production as shown by the 4 $\log_{10}$ reductions at 2 MOI

(Table 1) and the 8- and 116-fold reductions at 20 MOI, respectively (H-YK, data not shown). *Sanguisorbae radix* treatment induced a significant reduction in MHV-A59 production, as shown by the 31-fold reduction at 2 MOI (Table 1); however, no antiviral activity was detected at 20 MOI (H-YK, data not shown). *Pulsatillae radix* and *Punicae cortex* reduced MHV-A59 production by approximately five- and sixfold, respectively, at 2 MOI (Table 1); however, no reduction was detected at 20 MOI (H-YK, data not shown). *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* and *Torilis fructus* inhibited CoV production more strongly than ribavirin, which was used as a control (Table 1).

We also assessed the antiviral activity of these extracts on VSV, an unrelated virus, on other CoV, including MHV-JHM, the causative agent of encephalitis and demyelination in both rodents and primates, and PEDV [21]. MHV-A59 and MHV-JHM production was similarly attenuated by these extracts (Table 1). Consistent with the results obtained with the MHV-A59 strain, treatment with *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* or *Torilis fructus* inhibited MHV-JHM and PEDV production. *Punicae cortex* was an exception in that it reduced MHV-JHM and PEDV production by approximately 25-fold, but did not affect MHV-A59 production (Table 1). The inhibition of VSV was the least remarkable among the viruses tested (Table 1); however, *Torilis fructus* reduced VSV production by approximately 36-fold (Table 1), suggesting that *Torilis fructus* could be an effective broad-spectrum antiviral drug candidate.

Impaired intracellular MHV-A59 RNA synthesis following treatment with *Sophorae radix*, *Acanthopanacis cortex* or *Torilis fructus* was apparent at 5 h 30 min post-infection with 50 μg/ml of extract; this correlated approximately with the reduction in MHV production (Figure 1A). *Coptidis rhizoma* and *Moutan cortex radicis* were used for comparison with our previous results, which showed strong anti-CoV and almost no anti-CoV activities, respectively [15]. The results demonstrated that *Sophorae radix*, *Acanthopanacis cortex* and *Torilis fructus* significantly inhibited the expression of CoV RNA and, thus, subsequently inhibited both protein and virus production. Although virus production was reduced in the *Sanguisorbae-radix*-treated cells, MHV RNAs levels were similar in untreated and infected cells (Table 1 and Figure 1A), suggesting that *Sanguisorbae radix* might inhibit CoV protein expression and/or assembly and release.

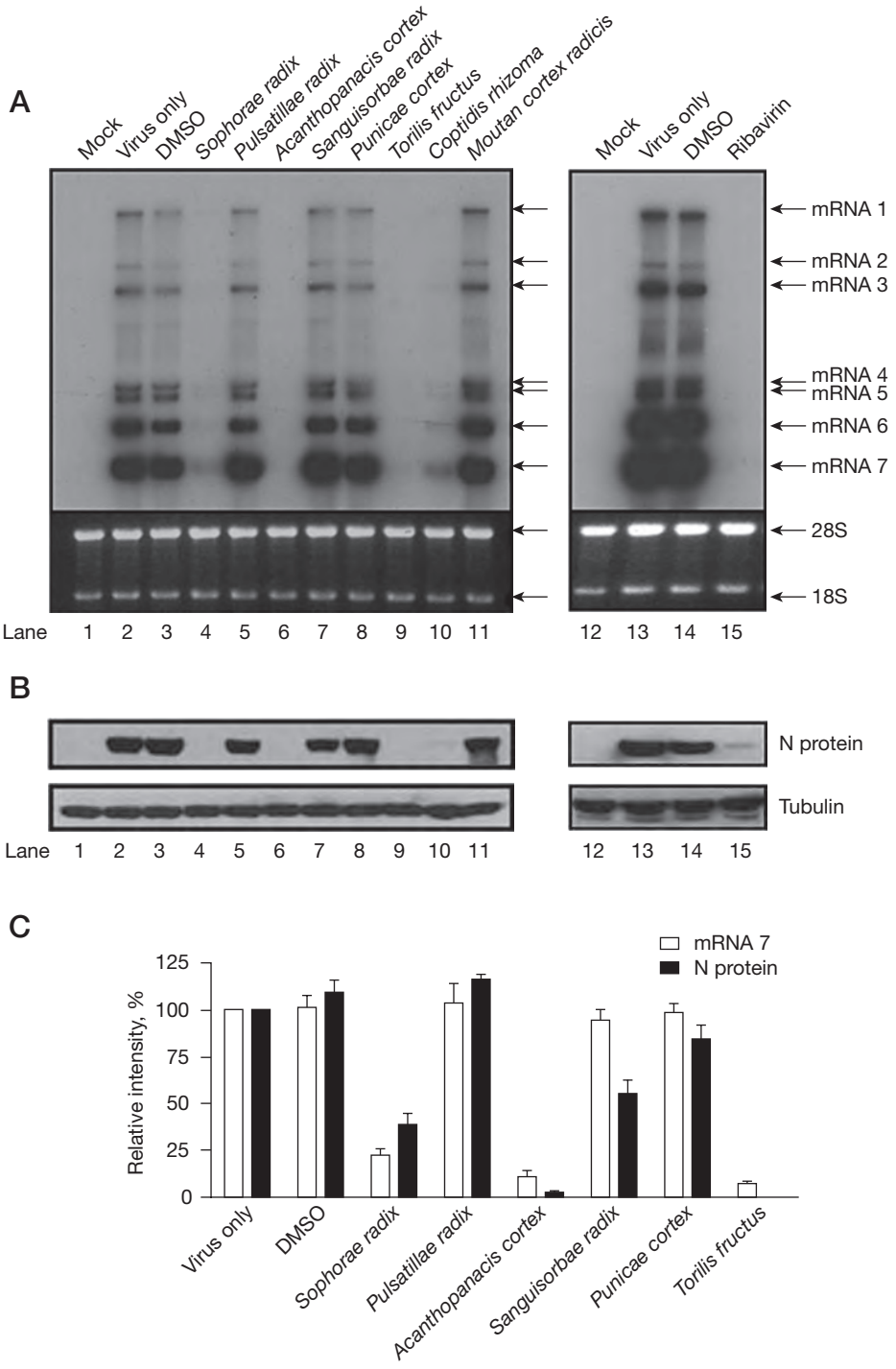
The effects of the herbal extracts on the expression of MHV N protein was assessed by western blot analysis using monoclonal antibodies against the N protein. N protein expression was markedly reduced by *Sophorae radix*, *Acanthopanacis cortex* and *Torilis fructus* extracts, which agreed with the decrease in MHV production and viral RNA synthesis (Figure 1A and 1B). In the cells treated with *Sanguisorbae radix*, MHV N protein expression was less than that observed in *the Punicae-cortex*-treated cells, although the MHV-A59 RNA levels were similar (Figure 1A and 1B); this suggests that the reduced virus production in the presence of *Sanguisorbae radix* extract was caused by the inhibition of viral protein synthesis, rather than the inhibition of virus assembly or release (Figure 1C). To determine the relative expression ratios of viral protein and RNA following extract treatment, the relative intensities of mRNA 7 and N protein (the most abudantly expressed viral mRNA and protein) were compared by densitometry (Image Gauge Version 4.0; Fuji Film Science Lab, 2001; Figure 1C). *Sophorae radix*, *Acanthopanacis cortex* and *Torilis fructus* treatments all reduced mRNA 7 (78%, 90% and 93% inhibition, respectively) and N protein synthesis (62%, 98% and 100% inhibition, respectively; Figure 1C). As mentioned previously, *Sanguisorbae radix* reduced N protein synthesis (45% inhibition) without eliciting any significant effect on mRNA 7 synthesis.

To analyse the inhibition of viral entry during MHV replication by the extracts, one set of DBT cells was infected with 2 MOI of MHV-A59 in the presence of 50 μg/ml extract during the viral adsorption period for 1 h at 4°C and then temperature shifted to 37°C, a temperature at which the viruses would be endocytosed in the presence of extracts. The DBT cells were incubated with extracts throughout the infection cycle. A second set of DBT cells was pre-adsorbed with MHV-A59 for 1 h at 4°C in the absence of extract and then the non-adsorbed viruses were washed out. Following this treatment, the MHV pre-adsorbed DBT cells were temperature shifted to 37°C and then further incubated with extracts at a concentration of 50 μg/ml until the completion of the experiment. If the second set of cells expressed higher levels of viral RNA than the first set of cells, it would indicate that the extract interfered with viral entry. In this experiment, more viral RNAs were expressed in the second set of *Sophorae-radix*- and *Torilis-fructus*-treated cells (Additional file 3), suggesting that viral entry is probably influenced, at least partly, by *Sophorae radix* and *Torilis fructus*.

Effects of selected extracts on MHV replication and cell viability

To determine the EC_{50} values, varying concentrations of each extract (1–100 μg/ml) were applied to DBT cells from the adsorption period to the end of the infection cycle. Irrespective of the extract, MHV production was reduced in a dose-dependent manner (Figure 2A). The mean ±SD EC_{50} values of *Sophorae radix*, *Pulsatillae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix*,

Figure 1. Effects of medicinal herbal extracts on MHV-A59 RNA and protein expression

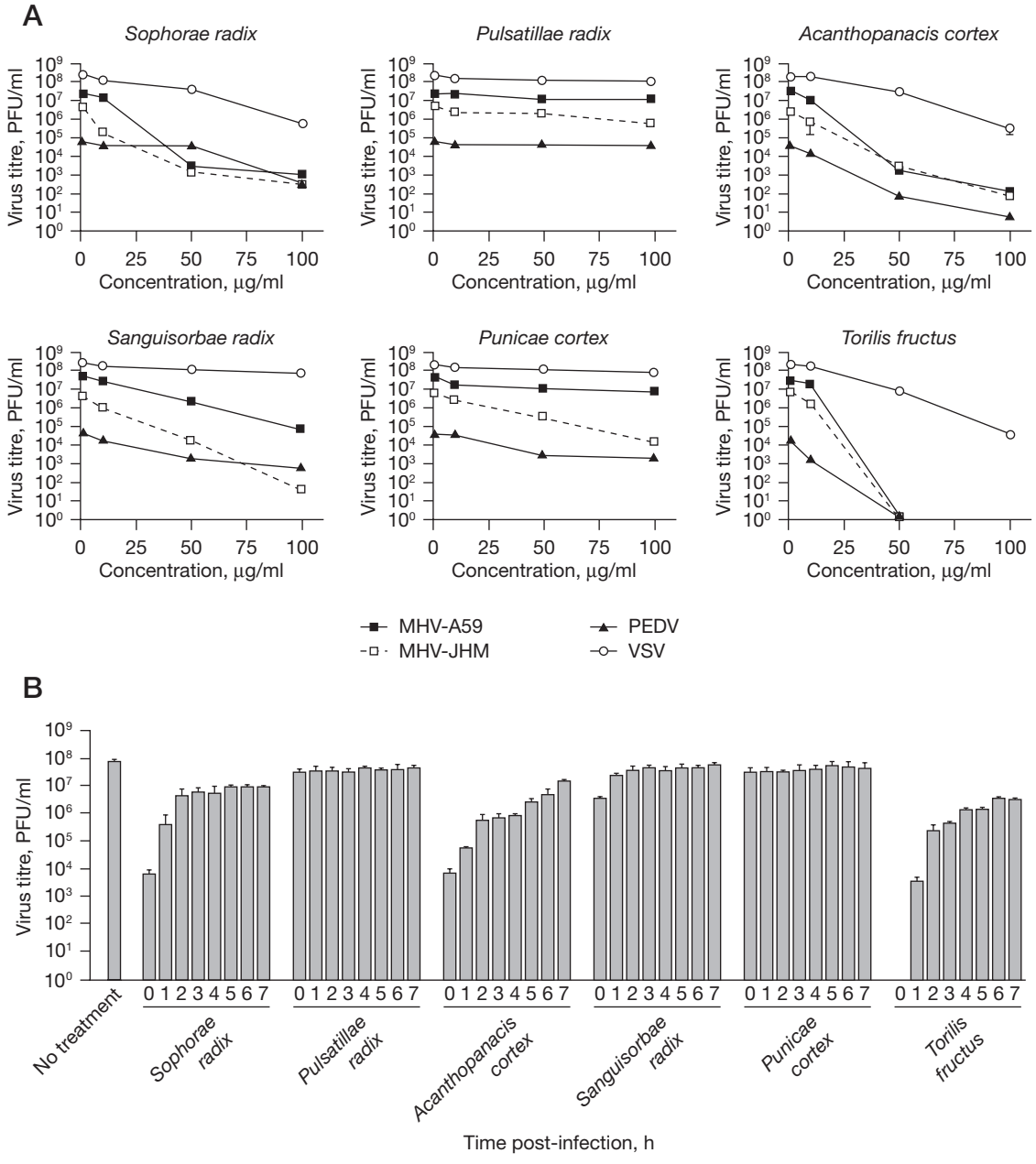


(A) Northern blot to detect mouse hepatitis virus (MHV) A59 strain (MHV-A59) RNA expression in response to treatment with 50 μg/ml of extract. Intracellular RNAs from mock-infected (mock; lanes 1 and 12), MHV-infected without extract treatment (virus only; lanes 2 and 13), MHV-infected with dimethyl sulfoxide (DMSO; lanes 3 and 14), MHV-infected with 50 μg/ml of ribavirin (lane 15), and MHV-infected and *Sophorae-radix-*, *Pulsatillae-radix-*, *Acanthopanacis-cortex-*, *Sanguisorbae-radix-*, *Punicae-cortex-*, *Torilis-fructus-*, *Coptidis-rhizoma-* and *Moutan-cortex-radicis*-treated cells (lane 4–11, respectively) were extracted at 5 h 30 min post-infection and analysed by northern blot analysis using a gene-7-specific probe. A 50 μg/ml aliquot of ribavirin, a nucleoside analogue that is widely used as an antiviral agent, was included as a positive control. *Coptidis rhizoma* and *Moutan cortex radicis* were also included for comparison with our previous results [15]. The messenger RNA (mRNA) and 28S and 18S ribosomal RNAs of seven species are also shown. **(B)** Western blot analysis to detect MHV-A59 nucleocapsid (N) protein in response to treatment with 50 μg/ml of extract. Lysates at 6 h post-infection were subjected to 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes and incubated with monoclonal antibodies against MHV N protein. **(C)** Quantitative analysis of the expression of mRNA 7 and N protein. Relative expression levels of mRNA 7 and N protein are presented by designating MHV-A59 infection only (lane 2 from [A] and [B]) as 100% by densitometry (Image Gauge version 4.0; Fuji Film, Tokyo, Japan). The mean ±SD of RNA expression and protein levels from three independent experiments are shown.

Punicae cortex and *Torilis fructus* were 0.8 ±0.2, 6.1 ±2.7, 0.9 ±0.1, 3.7 ±1.4, 11.0 ±0.8 and 0.8 ±0.0 μg/ml, respectively (Table 2). The mean ±SD EC_{90} values of *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* and *Torilis fructus* were 22.7 ±0.4, 14.8 ±1.1, 34.8 ±2.0 and 17.0 ±0.02 μg/ml, respectively.

We also determined the CC_{50} values of these extracts by the MTT-based cell viability assay. The CC_{50} was

Figure 2. Dose- and time-dependent inhibition of MHV-A59, MHV-JHM, PEDV and VSV replication by *Sophorae radix*, *Pulsatillae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix*, *Punicae cortex* and *Torilis fructus*



(A) Dose-dependent inhibition of mouse hepatitis virus (MHV) strains A59 (MHV-A59) and John Howard Mueller (MHV-JHM), porcine epidemic diarrhoea virus (PEDV) and vesicular stomatitis virus (VSV) replication. Varying concentrations (1, 10, 50 and 100 μg/ml) of six selected herbal extracts (*Sophorae radix*, *Pulsatillae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix*, *Punicae cortex* and *Torilis fructus*) were applied to DBT cells. The DBT cells were simultaneously infected with MHV-A59, MHV-JHM and VSV at a multiplicity of infection (MOI) of 2 or with PEDV at 0.5 MOI for 12 h at 37°C. The virus titres were measured using plaque assays. **(B)** Time-dependent inhibition of MHV-A59 replication. The six selected herbal extracts were applied to MHV-A59-infected DBT cells from 0 to 7 h post-infection at 37°C and were incubated until 12 h post-infection. The viral titre was measured using plaque assays. The error bars represent the standard deviations from three independent experiments. PFU, plaque-forming units.

defined as the concentration of extract that reduced cell viability to 50% of that of the control (cells untreated by extract). This yielded results that were in the range of mean ±SD 156.5 ±2.6 to 1,530.6 ±14.4 μg/ml (Table 2). The selectivity index (SI; CC_{50}/EC_{50}) for anti-MHV-A59 ranged from 38.8 to 696.0 (Table 2). Most of the cells were unaffected or only slightly affected when treated with 50 μg/ml of extract for up to 48 h. Although *Acanthopanacis-cortex*-treated cells showed a slight delay in doubling time the cells grew to 100% confluence.

The EC_{50} values of the extracts for MHV-JHM, PEDV and VSV were also determined (Additional file 4). The CC_{50} value for Vero cells was substantially higher than 200 μg/ml, with the exception of *Torilis fructus*, which was 152.5 ±2.2 μg/ml (Additional file 4), indicating that the DBT cells were very sensitive to the extracts. The EC_{50} values of the extracts for MHV-A59, MHV-JHM and PEDV were not very different (Table 2 and Additional file 4). The SIs for MHV-JHM, PEDV and VSV ranged from 31.9 to 293.1, 18.1 to >208.7 and 12.2 to >338.9, respectively (Additional file 4), suggesting that the extracts could be useful as broad-spectrum antiviral agents.

Table 1 and Figure 2A show the results of experiments in which the cells were treated with extract, and infected simultaneously with MHV. To evaluate the effects of the extracts on different stages of the viral replication cycle, we treated the cells with 50 μg/ml extract at the time of infection or at different time points (0–7 h), and then harvested the cells at 12 h post-infection. MHV-A59 production was reduced (Figure 2B), although to a lesser extent than when the cells were simultaneously exposed to MHV and treated with extract (Table 1 and Figure 2A). The antiviral activity of the extracts diminished as viral replication proceeded; hence, the loss of MHV production correlated with the time of extract treatment. These results were dependent upon the replication stage of the virus and the length of time it was exposed to the extract (Figure 2B). For all of the extracts, MHV production was reduced in a time-dependent manner. Treatment with *Sophorae radix*, *Pulsatillae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix*, *Punicae cortex* or *Torilis fructus* at 7 h post-infection reduced MHV production by 10-, 2-, 6-, 2-, 2- and 26-fold, respectively (Figure 2B).

Inhibitory effects of the extracts on the replication cycle of MHV-A59

To examine whether the herbal extract directly alters the virion, thereby preventing infection of the cells with MHV-A59, MHV-A59 virus particles were pretreated for 1 h with the extract and infection was then measured by titrating the released viruses. Most extracts, with the exception of *Torilis fructus*, did not show any direct effects on the viruses. MHV production was reduced by approximately 10-fold following pretreatment with *Torilis fructus* (Additional file 5A) suggesting that, in addition to its ability to reduce MHV-A59 RNA/protein expression (Figure 1A and 1B), *Torilis fructus* might also directly inhibit MHV-A59 infectivity. However, because virus production was completely abolished in MHV-A59-infected cells treated with *Torilis fructus* extract, the direct virucidal effect of the *Torilis fructus* extract, if it exists, is not a major activity that inhibits virus production. The direct inhibition on virion might be attributable to the altered virions, such as virion proteins or virion RNA. In an effort to exclude the latter possibility, we determined whether viral RNA isolated from *Torilis-fructus*-treated virions remained infectious. Mock-, DMSO-, *Pulsatillae-radix*- or *Torilis-fructus*-treated virion RNAs were extracted and subsequently transfected into DBT cells using lipofectin (Invitrogen). After 16 h of incubation at 37°C, virus production was analysed by a plaque assay (Additional file 5B). Regardless of extract treatment, virion RNA proved to be equally infectious, indicating that *Torilis fructus* does not alter virion RNA. Also, *Torilis fructus* did not decrease

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

Sample	MHV-A59		
	EC_{50}, μg/ml[a]	CC_{50}, μg/ml[b]	SI[c]
Sophorae radix	0.8 ±0.2	556.8 ±2.9	696.0
Pulsatillae radix	6.1 ±2.7	236.8 ±7.2	38.8
Acanthopanacis cortex	0.9 ±0.1	170.0 ±6.4	188.9
Sanguisorbae radix	3.7 ±1.4	388.4 ±4.5	105.0
Punicae cortex	11.0 ±0.8	1,530.6 ±14.4	139.1
Torilis fructus	0.8 ±0.0	156.5 ±2.6	195.6
Ribavirin	17.5 ±2.9	1,076.2 ±25.4	61.5

Each value represents the mean ±SD from three independent experiments. [a]The 50% effective concentration (EC_{50}) was determined as the concentration of extracts needed to inhibit the virus titre by 50% of the control value (cells without addition of extracts). [b]The 50% cytotoxic concentration (CC_{50}) was determined as the concentration of extracts necessary to reduce the cell viability to 50% of the control (cells without addition of extracts). [c]The selectivity index (SI) was calculated by CC_{50}/EC_{50}. MHV-A59, mouse hepatitis virus A59 strain.

viral RNA infectivity (Additional file 5B). This result further suggested that *Torilis fructus* might alter virion proteins. Mock-, DMSO-, *Pulsatillae-radix*- or *Torilis-fructus*-treated virion proteins were analysed by western blot analysis, which showed no degradation of major structural proteins, S, N and M (Additional file 5C). No RNA degradation was detected when *Torilis fructus* was incubated with virion RNA, indicating that *Torilis fructus* extract did not contain RNase activity (Additional file 5D). These results indicate that the extracts had antiviral activity rather than virucidal activity.

Effects of extracts on intracellular signalling pathways of cells with or without MHV-A59 infection

It has been previously reported that curcumine derived from *Curcuma longa* extract inhibits MHV-A59 replication by inhibiting the activity of COX, a prostaglandin H_2 synthase that converts arachidonic acid into prostaglandin [22]. Prostaglandins are associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular diseases, and colon cancer [23]. Two isoforms of COX (COX-1 and COX-2) have been identified. In contrast to COX-1, which is constitutively expressed in almost all tissues, COX-2 shows a low basal level of expression and is induced by inflammation, other physiological stimuli and growth factors.

To test whether the selected extracts might inhibit the replication of MHV-A59 by affecting the inflammatory responses in the infected cells, we evaluated the expression of COX-2 mRNA. The results of RT-PCR revealed that if the cells were infected with MHV-A59, COX-2 mRNA levels were higher in the *Acanthopanacis-cortex*- and *Torilis-fructus*-treated cells (Figure 3A, lanes 10 and 16, and Figure 3B) than in untreated cells. As controls, cells were infected and uninfected with MHV-A59 in the absence and presence of phorbol myristate acetaste (PMA), a COX-2 inducer (Figure 3A, lanes 17–20). COX-2 mRNA levels were also higher if MHV-A59-infected cells were treated with PMA (Figure 3A, lane 20).

To quantify COX-2 mRNA levels in infected and/or extract-treated cells, real-time RT-PCR was performed (Figure 3B). When the cells were infected with MHV-A59 in the absence of extracts, COX-2 mRNA levels increased (Figure 3B). In uninfected cells, COX-2 mRNA levels were increased by *Acanthopanacis cortex* and *Torilis fructus* (Figure 3B). These results indicated that MHV-A59 infection or treatment with *Acanthopanacis cortex* or *Torilis fructus* extract can induce COX-2 expression. In the cells simultaneously infected with MHV-A59 and treated with *Acanthopanacis cortex* or *Torilis fructus*, COX-2 mRNA levels were even more significantly increased (Figure 3B). These results suggest that the antiviral activities of *Acanthopanacis cortex* and *Torilis fructus* extracts could be attributed to increased COX-2 mRNA expression. By contrast, the effects of *Sophorae radix* and *Sanguisorbae radix* might not be directly related to increased or decreased COX-2 mRNA expression (Figure 3B, lanes 6 and 12).

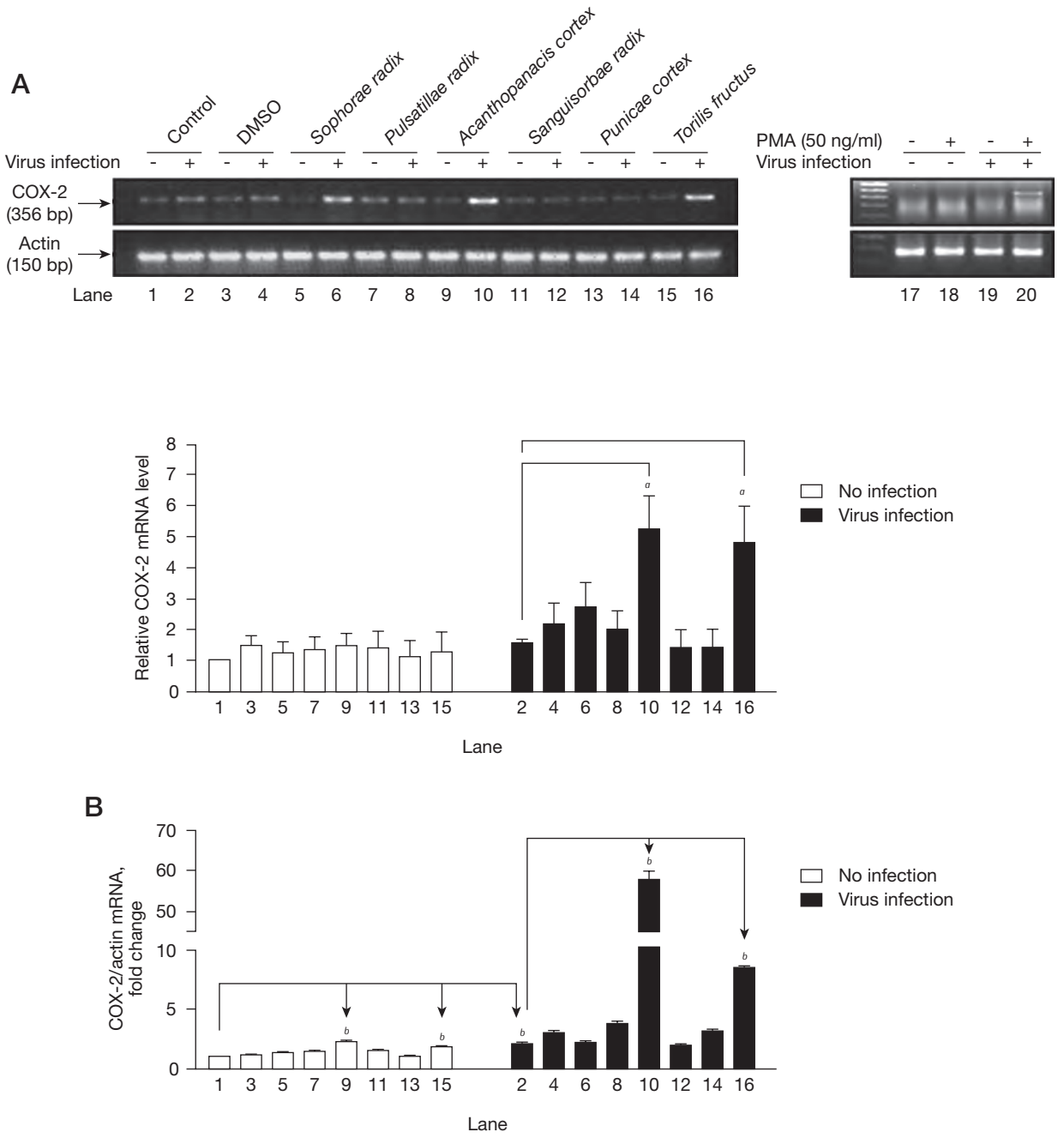
To further evaluate the involvement of intracellular signalling pathways and COX-2 expression in *Acanthopanacis-cortex*- and *Torilis-fructus*-treated cells, we performed western blot analyses to measure the expression and phosphorylation of ERK1/2, JNK and p38 in cells simultaneously infected with MHV-A59 and treated with extract. Phosphorylated ERK1/2 levels were increased in cells simultaneously infected with MHV-A59 and treated with *Acanthopanacis cortex* or *Torilis fructus* extract (Figure 3C, lanes 10 and 16). Phosphorylated p38 levels were increased in the *Acanthopanacis-cortex*-treated cells, but not in the *Torilis-fructus*-treated cells (Figure 3C, compare lanes 10 and 16). The extracts did not affect the expression or phosphorylation of JNK in MHV-A59-infected cells. Accordingly, we hypothesized that when cells were infected with MHV-A59, *Acanthopanacis cortex* increased COX-2 expression via ERK1/2 and p38 activation, whereas *Torilis fructus* increased COX-2 expression via ERK1/2 activation (Figure 3C). Although we were unable to demonstrate any direct correlation between anti-MHV-A59 activity and induced COX-2 expression, we propose that *Acanthopanacis cortex* and *Torilis fructus* might exert anti-CoV activity, at least in part, through inflammatory responses occurring as a result of COX-2 expression induced by the activation of ERK1/2 and/or p38.

We also performed real-time RT-PCR of proinflammatory cytokines, such as IL-6 and TNF-α. In MHV-A59-infected cells, mRNA expression levels of IL-6 and TNF-α were increased (Figure 3D and 3E, lane 1 versus 2). In cells simultaneously infected with MHV-A59 and treated with *Sophorae radix*, *Sanguisorbae radix*, *Punicae cortex* or *Torilis fructus*, the mRNA expression level of IL-6 was decreased (Figure 3D, lanes 2 versus 6, 12, 14 or 16), demonstrating that the antiviral activities of these extracts decreased proinflammatory cytokine mRNA expression. TNF-α mRNA expression was decreased in the cells simultaneously infected with MHV-A59 and treated with *Sophorae radix* or *Torilis fructus* (Figure 3E, lanes 2 versus 6 or 16). However, IL-6 or TNF-α mRNA expression remained high or increased in infected cells treated with *Pulsatillae radix* or *Acanthopanacis cortex* (Figure 3D and 3E, lane 2 versus 8 or 10).

Discussion

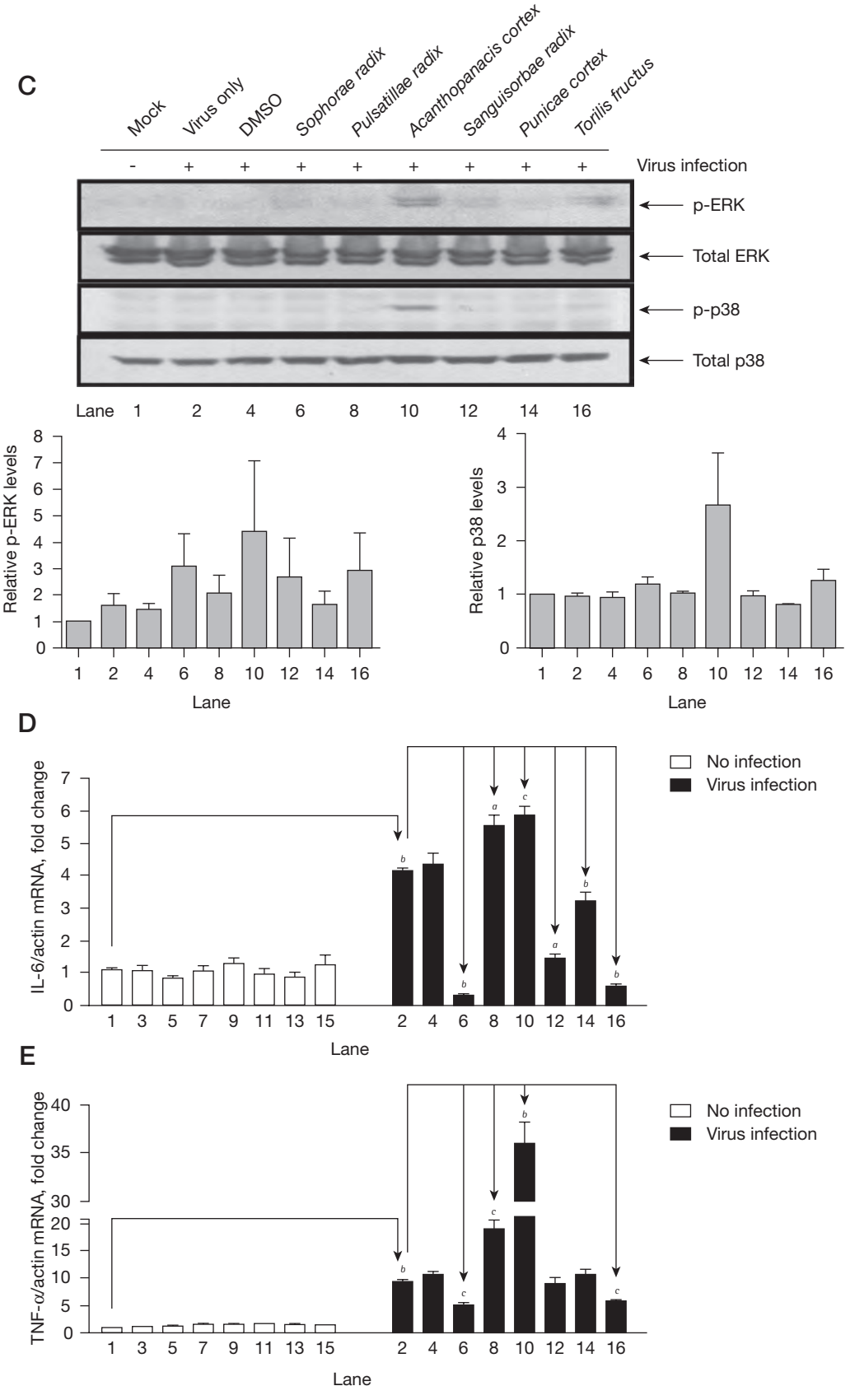
Many herbal extracts have been reported to have profound antiviral activity, and some of them have already been employed for the treatment of animals

Figure 3. Inhibition of MHV-A59 replication and cellular signalling pathways by extract treatment



(A) Reverse transcriptase (RT)-PCR was used to detect cyclooxygenase (COX)-2 messenger RNA (mRNA) expression in total RNA. A representative gel from three independent experiments is shown. The results of extract-treated (50 μg/ml; lanes 5–16) or phorbol myristate acetate (PMA)-treated (50 μg/ml; lanes 18 and 20) DBT cells uninfected (odd numbers up to lane 17 plus lane 18) or infected with mouse hepatitis virus (MHV) A59 strain (MHV-A59; even numbers up to lane 16 plus lanes 19 and 20) at 5 h 30 min post-infection are shown. Untreated DBT cells that were uninfected (lanes 1 and 3) and MHV-A59-infected (lanes 2 and 4) are also shown. The relative COX-2 mRNA expression level as compared with that of actin is presented. The level of COX-2 mRNA expression in cells that were uninfected and untreated was designated as 1 by densitometry (Image Gauge version 4.0; Fuji Film, Tokyo, Japan). The mean ±SD COX-2 mRNA expression from three independent experiments is shown. **(B)** Quantitative analysis of COX-2 mRNA expression. Total RNA was prepared as described in (A) and analysed by real-time RT-PCR. The numbering of each lane is the same as in (A). **(C)** Extracellular signal-related kinase (ERK)1/2, p38 and c-Jun N-terminal kinase (JNK) activation in 50 μg/ml of extract- or PMA-treated DBT cells with or without MHV-A59 infection at 5 h 30 min post-infection. After 5 h 30 min post-infection, the cells were lysed and equivalent amounts of total cell lysates were analysed by western blotting for total and active phosphorylated kinase (ERK)1/2 (p-ERK; p44/p42), total and active p38 (p-p38), and total and active JNK. **(D)** Quantitative analysis of interleukin (IL)-6 mRNA expression. Total RNA was prepared as described in (A) and analysed by real-time RT-PCR. The numbering of each lane is the same as in (A). **(E)** Quantitative analysis of tumour necrosis factor (TNF)-α mRNA expression. Total RNA was prepared as described in (A) and analysed by real-time RT-PCR. The numbering of each lane is same as in (A). [a]$P<0.05$ versus control. [b]$P<0.001$ versus control. [c]$P<0.01$ ($n=5$). bp, base pair; DMSO, dimethyl sulfoxide.

Figure 3. Continued



and people suffering from viral infections, such as herpes simplex virus and HBV infections [24,25]. Since the SARS outbreak, many attempts have been made to find treatments for CoV infections [6–8]. Among them, several components of traditional Chinese medicine (TCM) and many small molecules of herbal origin have been shown to exhibit some anti-SARS-CoV activities *in vitro* – which explains, in part, the beneficial effects of TCM observed in SARS patients [9,10,14,26]; however, the antiviral mechanisms of these herb-originated compounds remain to be clearly elucidated. Glycyrrhizin (the active compound from licorice roots), which inhibited SARS-associated CoV replication [9,27], has been shown to be involved in a variety of cellular signalling pathways [28], although the mechanism underlying the antiviral effects of glycyrrhizin has yet to be clearly elucidated.

Throughout the life cycle of CoV, there are several potential targets for antiviral agents that interfere with 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 a previous report, we suggested that *Cimicifuga rhizome*, *Meliae cortex*, *Coptidis rhizome* or *Phellodendron cortex* might mainly inhibit RNA-dependent RNA polymerase or protease activity that is crucial for CoV RNA replication because viral entry was not the main target and viral RNA synthesis was decreased substantially [15]. We suggest that the antiviral mechanisms of the extracts used in the present study are similar to those described in our previous report because viral RNA synthesis was substantially reduced in *Sophorae radix*-, *Acanthopanacis-cortex*- or *Torilis-fructus*-treated cells (Figure 1A and Additional file 3). However, in *Sophorae-radix*- and *Torilis-fructus*-treated cells, we suggest that viral entry might also be influenced, at least in part (Additional file 3).

In the *Sanguisorbae-radix*-treated cells, MHV N protein expression was reduced (45% inhibition) without any significant reduction in mRNA 7 synthesis, whereas virus production was reduced by 31-fold (Table 1 and Figure 1); this suggests that decreased virus production as a result of *Sanguisorbae radix* treatment might be attributable to the inhibition of viral protein synthesis.

Sophorae radix, the dried root of *Sophora flavescens Aiton*, which contains matrine as an active compound, has been used in TCM for the treatment of asthma, allergy, atherosclerosis and arrhythmia [29–32]. Additionally, the aqueous extract of *Sophorae radix* showed anti-HBV activity *in vitro* and protected mice against liver damage *in vivo* [33]. *Acanthopanacis cortex*, which contains acanthoside, chiisanoside and phytosterol as active compounds, has been commonly employed as an anti-inflammatory agent, such as for the treatment of rheumatoid arthritis, anti-oxidation, anti-tumour, the promotion of immunoregulation and relief of anxiety and depression [34]. *Sanguisorbae radix*, which contains sanguiin H-6 as its main active compound, exhibits nitric oxide production-suppressing activity during infection, inflammation or immune responses [35], and has been utilized for haemostasis. *Punicae cortex* exhibits anti-oxidant, anti-carcinogenic and anti-inflammatory properties [36]. *Torilis fructus* contains torilin, which exhibits profound anti-tumour activity as one of its effective components [37].

COX-2 gene expression is regulated at both the transcriptional and post-transcriptional levels [38]. Several viruses, such as Epstein–Barr virus (EBV), HIV and HBV are capable of stimulating COX-2 via a broad range of cellular signal transduction cascades, including Ras-Raf-mitogen-activated protein kinase, JNK, nuclear factor-κB and Janus kinase signal transducers and activators of transcription signalling pathways [39,40].

Unlike curcumine derived from *Curcuma longa* extract, which inhibits MHV-A59 replication by inhibiting COX-2 activity, our results showed that COX-2 expression in MHV-A59-infected cells was increased in *Acanthopanacis-cortex*- and *Torilis-fructus*-treated cells. ERK1/2 and p38 were phosphorylated and COX-2 expression was significantly increased in cells infected with MHV-A59 and treated with *Acanthopanacis-cortex* (Figure 3A and 3B). p38 was phosphorylated and COX-2 expression was significantly increased in cells infected with MHV-A59 and treated with *Torilis fructus* (Figure 3). Because MHV-A59 replication was decreased significantly and COX-2 expression was induced in *Acanthopanacis-cortex*- and *Torilis-fructus*-treated cells, we hypothesized that up-regulated COX-2 expression occurring via the activation of ERK1/2 and/or p38 might be involved, at least partly, in antiviral activity. The levels of proinflammatory cytokines, such as IL-6 and TNF-α, increased in MHV-A59-infected and *Acanthopanacis-cortex*-treated cells (Figure 3D and 3E, lane 10), suggesting that the increased cytokine expression levels played a role in the antiviral activities. From this, we further hypothesize that the increased COX-2 expressions in *Acanthopanacis-cortex*- and *Torilis-fructus*-treated cells might induce different inflammatory mediators to inhibit MHV-A59 replication.

In summary, the results of the present study showed that *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* and *Torilis fructus* extracts reduced the production of CoV *in vitro*. *Punicae cortex* treatment reduced the productions of MHV-JHM and PEDV, but not of MHV-A59. In particular, *Torilis fructus* could be a candidate broad-spectrum inhibitor because VSV was also reduced by treatment with *Torilis fructus*. These data demonstrate that the screened herbal extracts could be candidate inhibitors of CoV production

via their inhibitory effects on replication steps and/or their effects on cellular signal transduction pathways. Accordingly, we suggest that the selected herbal extracts have antiviral activity that could be used to treat CoV infections in both humans and animals. Although further studies will be required to elucidate the antiviral mechanism of the extracts at the molecular level in greater detail, our results indicate that the medicinal herbal extracts of *Sophorae radix*, *Acanthopanacis cortex*, *Sanguisorbae radix* or *Torilis fructus* might be potential candidates for the development of an anti-CoV agent and/or various antiviral drugs.

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Disclosure statement

The authors declare no competing interests.

Additional files

Additional file 1: A supplementary file showing the extracts tested and relative MHV-A59 productions can be found at http://www.intmedpress.com/uploads/documents/AVT-09OA-1474_Kim_Add_file1.pdf

Additional file 2: A supplementary file showing the primers used for real-time RT-PCR can be found at http://www.intmedpress.com/uploads/documents/AVT-09OA-1474_Kim_Add_file2.pdf

Additional file 3: A supplementary file showing the inhibitory effects of extracts on the replication cycle of MHV-A59 can be found at http://www.intmedpress.com/uploads/documents/AVT-09OA-1474_Kim_Add_file3.pdf

Additional file 4: A supplementary file showing the effects of six medicinal herbal extracts on MHV-JHM, PEDV and VSV can be found at http://www.intmedpress.com/uploads/documents/AVT-09OA-1474_Kim_Add_file4.pdf

Additional file 5: A supplementary file showing the direct effects of extracts on MHV-A59 can be found at http://www.intmedpress.com/uploads/documents/AVT-09OA-1474_Kim_Add_file5.pdf

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