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Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13

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ABSTRACT

Severe acute respiratory syndrome (SARS) is an infectious disease with a strong potential for transmission upon close personal contact and is caused by the SARS-coronavirus (CoV). However, there are no natural or synthetic compounds currently available that can inhibit SARS-CoV. We examined the inhibitory effects of 64 purified natural compounds against the activity of SARS helicase, nsP13, and the hepatitis C virus (HCV) helicase, NS3h, by conducting fluorescence resonance energy transfer (FRET)-based double-strand (ds) DNA unwinding assay or by using a colorimetry-based ATP hydrolysis assay. While none of the compounds, examined in our study inhibited the DNA unwinding activity or ATPase activity of human HCV helicase protein, we found that myricetin and scutellarein potently inhibit the SARS-CoV helicase protein in vitro by affecting the ATPase activity, but not the unwinding activity, nsP13. In addition, we observed that myricetin and scutellarein did not exhibit cytotoxicity against normal breast epithelial MCF10A cells. Our study demonstrates for the first time that selected naturally-occurring flavonoids, including myricetin and scultellarein might serve as SARS-CoV chemical inhibitors.

SARS is an atypical pneumonia, primarily transmitted by respiratory droplets or personal contacts. SARS was an epidemic illness that occurred between 2002 and 2003, and caused more than 700 deaths around the world (more information can be found at http://www.who.int/csr/sars/en). Since the first diagnosis in Guangdong province, China, successive outbreaks occurred in 29 countries and about 20% of the patients inflicted with the SARS virus eventually developed the symptoms of acute respiratory distress syndrome (ARDS), which required a mechanical ventilation support for survival. 50% of the patients who developed ARDS eventually passed away, although the mortality varied, depending on age.[1] In addition, the rapid spread of SARS did not allow for controlled clinical treatments during the outbreak and, therefore, empirical strategies were employed to treat patients with such agents as antiviral drugs, steroids, and type-I interferons; however, in a retrospective review of the literature, none of the medications actually benefited patients.[2] Therefore, there is a need to develop effective anti-SARS viral agents in the event of a future SARS outbreak.

SARS-CoV was isolated and shown to be a class of coronavirus that is a single stranded RNA virus with a genome of 29,751 bases. Based on the genome sequence, the SARS-CoV was found to be only moderately related to other human coronaviruses, HCoV-OC43 and HCoV-229E, and did not resemble any of the three previously known groups of coronaviruses.[3] Coronaviruses are members of a family of enveloped viruses that replicate in the cytoplasm of animal host cells. Upon infection of target cells, the genome of SARS-CoV is translated into two large replicative polyproteins that are subsequently processed into a number of non-structural proteins (nsPs) by the viral protease.[4] These nsPs include the RNA-dependent RNA polymerase and the helicase. Since the viral helicase is essential to viral genome replication, it is currently considered a potential target for anti-viral drug development.

The present study was conducted to identify natural compounds that might inhibit SARS-CoV helicase activity in vitro. In order to accomplish this goal, we prepared natural chemical stocks (Table 1) and examined their effects on the activity of SARS-CoV helicase, nsP13.[5] Although SARS-CoV contains a RNA-dependent RNA polymerase, nsP13 has been reported to possess dsDNA

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Table 1
List of natural compounds used in our study

No	Compound	Source	No	Compound	Source	No	Compound	Source
1	Daidzin	Chromadex	22	Isoliquiritigenin	*Glycyrrhiza glabra*	43	Ursolic acid	*Bridelia cambodiana*
2	Isohesperidin	Chromadex	23	1-Isomangostin	*Garcinia mangostana*	44	Oleanolic acid	*Bridelia cambodiana*
3	Galangin	Chromadex	24	γ-Mangostin	*Garcinia mangostana*	45	Stigmasterol	*Bridelia cambodiana*
4	Sophoricoside	Chromadex	25	α-Mangostin	*Garcinia mangostana*	46	β-Sitosterol	*Bridelia cambodiana*
5	Isoquercetin	Chromadex	26	Lambertiainc acid	*Thuja orientalis*	47	Daucosterol	*Bridelia cambodiana*
6	Myricetin	Chromadex	27	Perviridamide	*Aglaia perviridis*	48	Gypenoside XVII	*Panax ginseng*
7	Myricitrin	Chromadex	28	4-Hydroxypy ramidatine	*Aglaia perviridis*	49	Ginsenoside Rb1	*Panax ginseng*
8	Scutellarein	*Scutettaria baicalensis*	29	Pyramidatine	*Aglaia perviridis*	50	Imperatorin	*Saposhnikovia divaricata*
9	Chrysin	Chromadex	30	Verproside	*Phseudolysimachion longifolium*	51	Hamaudol	*Saposhnikovia divaricata*
10	Silymarin	Chromadex	31	Isovanillyl Catapol	*Phseudolysimachion longifolium*	52	3-*O*-Angeloylh amaudol	*Saposhnikovia divaricata*
11	Icaritin	Chromadex	32	6-*O*-Veratroyl Catalpol	*Phseudolysimachion longifolium*	53	5-*O*-Methylvisamminol	*Saposhnikovia divaricata*
12	Curcumin	Chromadex	33	Minecoside	*Phseudolysimachion longifoiium*	54	Ledebouriellol	*Saposhnikovia divaricata*
13	Scutellarin	*Scutellaria baicalensis*	34	Diosmetin-7-*O*-Glc	*Phseudolysimachion longifoiium*	55	Gallic acid	Chromadex
14	Baicalein	*Scutellaria baicalensis*	35	Diosmetin-7-*O*-Glc-Xyl	*Phseudolysimachion longifoiium*	56	Methoxyeugenol	*Cinnamomum cambodianum*
15	Hyperoside	Chromadex	36	3β-Friedelanol	*Bridelia cambodiana*	57	Spatulenol	*Thyrsanthera suborbicularis*
16	Naringin	Chromadex	37	Friedelin	*Bridelia cambodiana*	58	Taraxerol	*Thyrsanthera suborbicularis*
17	Naringenin	Chromadex	38	24-Methyllanosta-9(11),25-dien-3-one	*Bridelia cambodiana*	59	19-Hydroxy-1(10),15-rosadiene	*Thyrsanthera suborbicularis*
18	Amentoflavone	Chromadex	39	24,24-Dimethylla nosta-9(11),25-dien-3-one	*Bridelia cambodiana*	60	Aleuritolic acid	*Thyrsanthera suborbicularis*
19	Populnetin	Chromadex	40	24-Methyl-S«-lanosta-9 (11),25-dien-3oi-one	*Bridelia cambodiana*	61	Marliolide	*Cinnamomum cambodianum*
20	Icariin	Chromadex	41	Betulinic acid	*Bridelia cambodiana*	62	Sec-*O*-Glucosylha maudol	*Saposhnikovia divaricata*
21	8-Deoxygartanin	*Garcinia mangostana*	42	α-Amyrin	*Bridelia cambodiana*	63	4′-*O*-β-D-glucosyl-5-O-m ethylvisamminol	*Saposhnikovia divaricata*
						64	Prim-*O*-Glucosylcimifugin	*Saposhnikovia divarkata*

unwinding activity as well as the ability to translocate along the nucleic acids by hydrolyzing ATP.[6] We first attempted to screen compounds that suppress the DNA unwinding activity of nsP13 and the dsDNA unwinding activity of nsP13 was measured using a fluorometric assay, based on the FRET from the fluorescein to the carboxytetramethylrhodamine (TAMRA) (Fig. 1A).[7] FRET describes an energy transfer mechanism between two dye molecules, in which energy is transferred from a donor molecule to an acceptor molecule. This approach is highly useful in determining a dynamic interaction between two adjacent molecules. More specifically, our experimental setup was devised in such a way that FRET occurred from the fluorescein to TAMRA; thus, no fluorescence from fluorescein was generated when the two DNA strands were base-paired, but a strong fluorescence was generated and detectable due to the absence of FRET between fluorescein and TAMRA when the duplex was unwound by the nsP13 helicase. Based on this principle, we added individual natural compounds at a concentration of 10 μM to the the dsDNA-unwinding reaction and measured the emitting fluorescent intensity at a wavelength of 535 nm. In these experiments, none of the natural chemicals inhibited the dsDNA-unwinding activity of SARS helicase, nsP13 (Fig. 1B). In an identical experimental setup, we attempted to identify chemical inhibitors of the HCV viral helicase, NS3h, and found that none of the natural chemicals in our experiment inhibited the DNA unwinding activity of HCV viral helicase in vitro (Fig. 1C).

We then assessed whether any of these natural compounds could inhibit the ATPase activity of nsP13.[8] The ATP hydrolysis assay was conducted with nsP13 in the presence of M13 single-stranded (ss) DNA. M13 ssDNA is a 7,250 base long circular DNA that has no end and, therefore, the helicase is expected to continuously translocate along the ssDNA unless the helicase separates from the DNA. ATP hydrolysis was assessed using a colorimetric assay by measuring the release of P_i through the formation of the molybdate complex (Fig. 2A). Using this experimental setup, we examined whether there were any natural compounds that inhibited the ATP hydrolysis activity of nsP13 and found that out of the 66 natural chemicals tested, myricetin (No. **6**) and scutellarein (No. **8**) inhibited the ATPase activity of nsP13 by more than 90% at a concentration of 10 μM, while a few compounds such as myricitrin (No. **7**), amentoflavone (No. **18**), diosmetin-7-*O*-Glc-Xyl (No. **35**) and taraxerol (No. **58**) exhibited some degree of inhibition (around 20%), as shown in Figure 2B. Again, we were not able to detect any compounds in our natural compound library that suppressed the ATPase activity of the HCV viral helicase (Fig. 2C). In order to determine the IC_{50} value of **6** and **8** (Fig. 3A) in suppressing nsP13 ATPase activity, we serially diluted **6** and **8** and measured their inhibitory effects on the ATPase activity of nsP13 in vitro. As a result of this analysis, IC_{50} values of **6** and **8** were determined to be 2.71 ± 0.19 μM and 0.86 ± 0.48 μM, respectively (Fig. 3B). To determine whether myricetin or scutellarein possesses potential cytotoxicity in normal cells, we have exposed normal breast epithelial MCF10A cells to myricetin (2 μM) or scutellarein (2 μM) and observed whether they could exhibit inhibitory effects on the growth of MCF10A cells.[9] As a result, we observed that either myricetin or scutellarein did not affect the growth of MCF10A cells at cellular concentrations close to the IC_{50} of myricetin or scutellarein (Fig. 3C), suggesting that both myricetin and scutellarein are safe compounds at pharmacologically-effective concentrations.

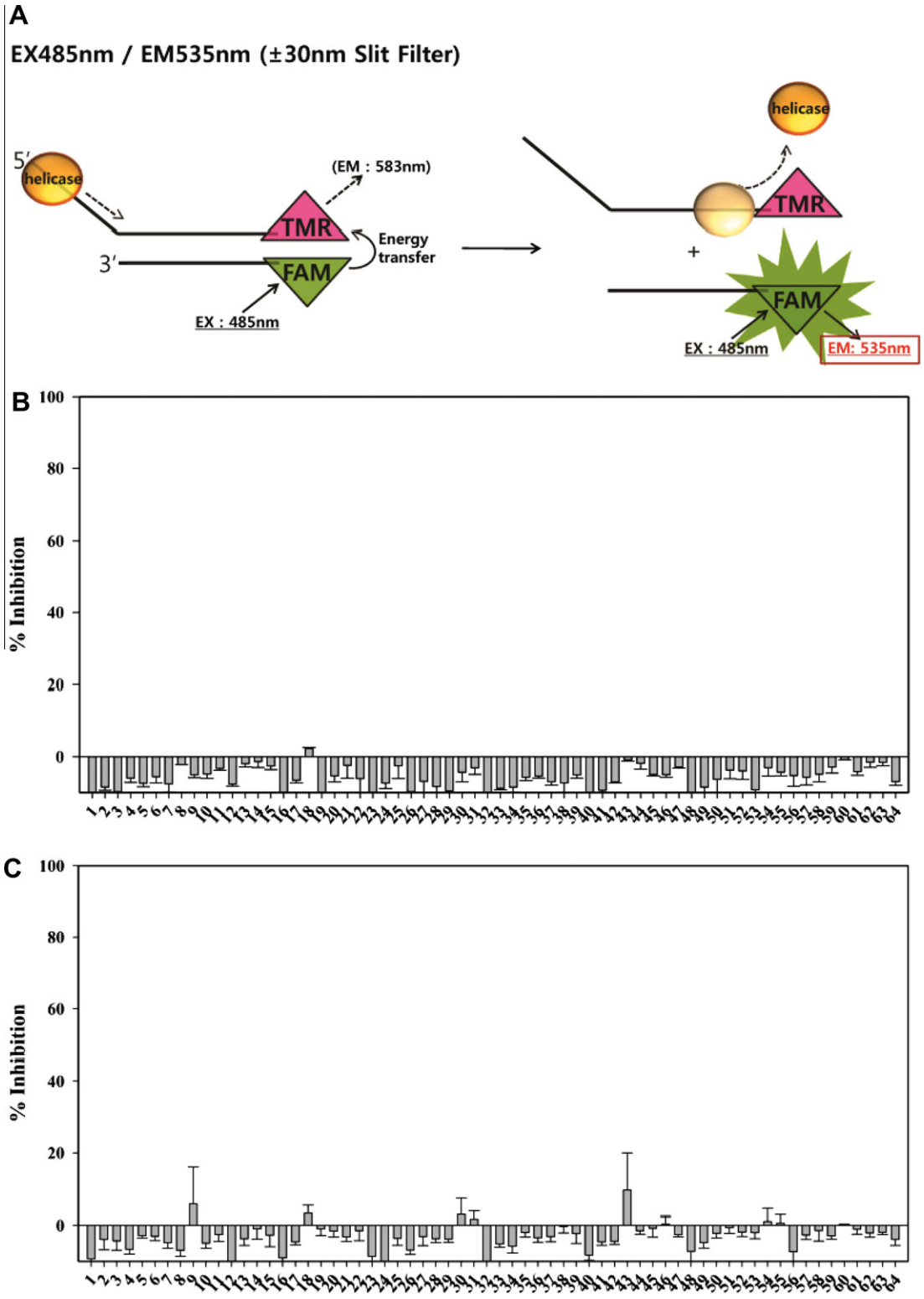

Figure 1. (A) Schematic representation of FRET-based dsDNA unwinding assay. (B) Inhibition of the dsDNA unwinding activity of the SARS CoV helicase in the presence of 10 μM natural compounds. (C) Inhibition of the dsDNA unwinding activity of the HCV helicase in the presence of 10 μM natural compounds.

Naturally-occurring chemicals are regarded as a great source of potential medications against various diseases. In particular, they have gained great scientific interest due to their strong neuroprotective, cardioprotective and chemopreventive activities. In addition, previous studies have demonstrated that selected naturally-occurring flavonoids exhibit anti-viral activities. For example, administration of silymarin, which is rich in milk thistle, significantly suppressed hepatitis B virus (HBV)-related hepatocarcinoma in HBV transgenic mice.[10] Quercetin also inhibited HCV production in an HCV cell culture system.[11] Epigallocatechin-3-gallate (EGCG), the major active constituent of green tea, suppressed human immunodeficiency virus (HIV) replication by degrading a

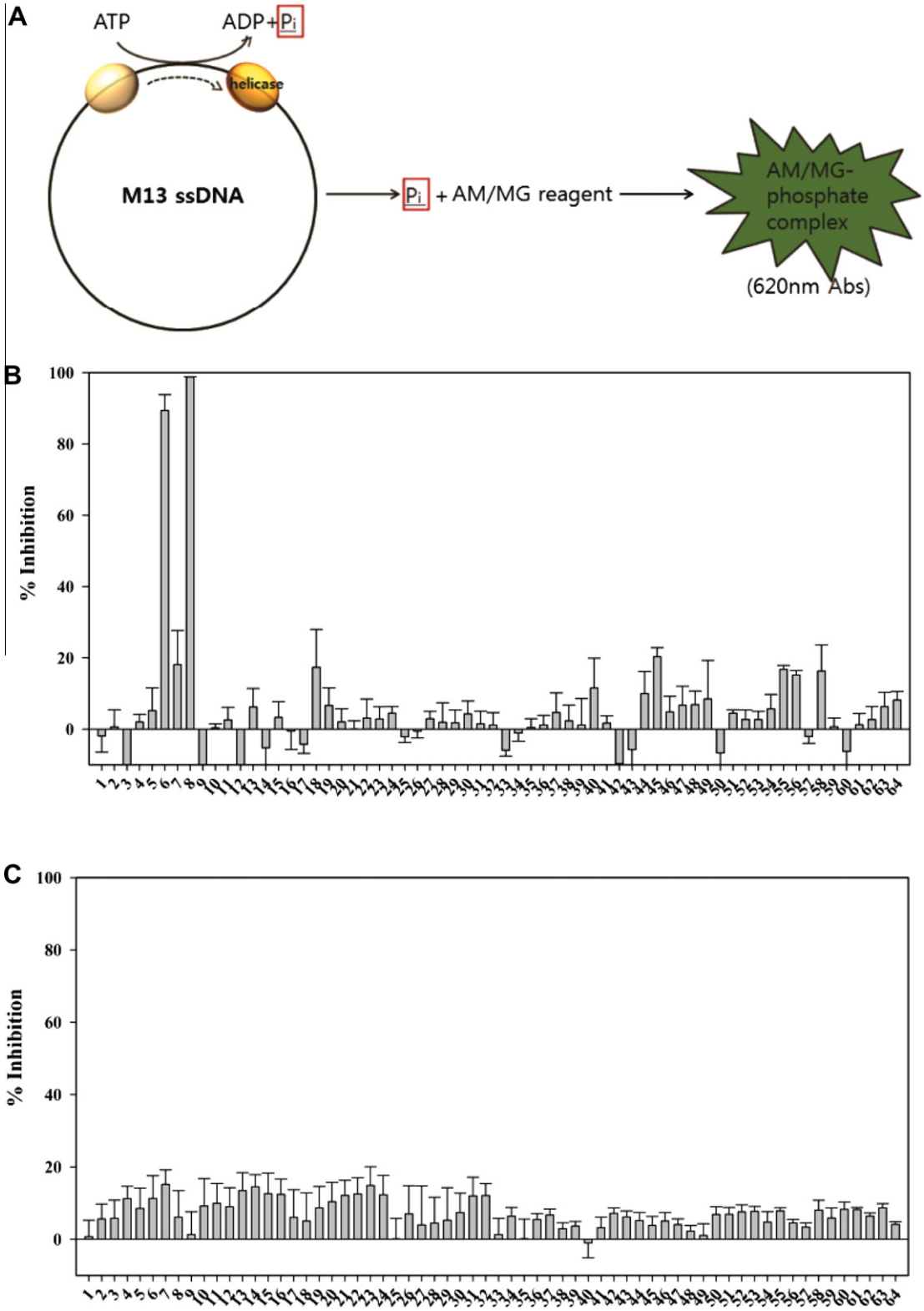

Figure 2. (A) Schematic representation of the ATP hydrolysis assay. (B) Inhibition of the ATP hydrolysis activity of the SARS CoV helicase in the presence of 10 μM natural compounds. (C) Inhibition of the ATP hydrolysis activity of the HCV helicase in the presence of 10 μM natural compounds

semen-derived enhancer of virus infection (SEVI), which is required for HIV virus infection.[12] Glycyrrhizin, an active ingredient in liquorice root, inhibited a SARS-associated virus in vero cells, although its clinical efficacy against the SARS virus in patients requires further verifications.[13] In the present study, we present the evidence for the first time that myricetin and scutellarein are strong chemical inhibitors of SARS-CoV helicase and this effect is mediated through inhibition of ATPase activity, but not inhibition of helicase activity. On the other hand, myricetin and scutellarein did not suppress the helicase activity of HCV virus in our experi-

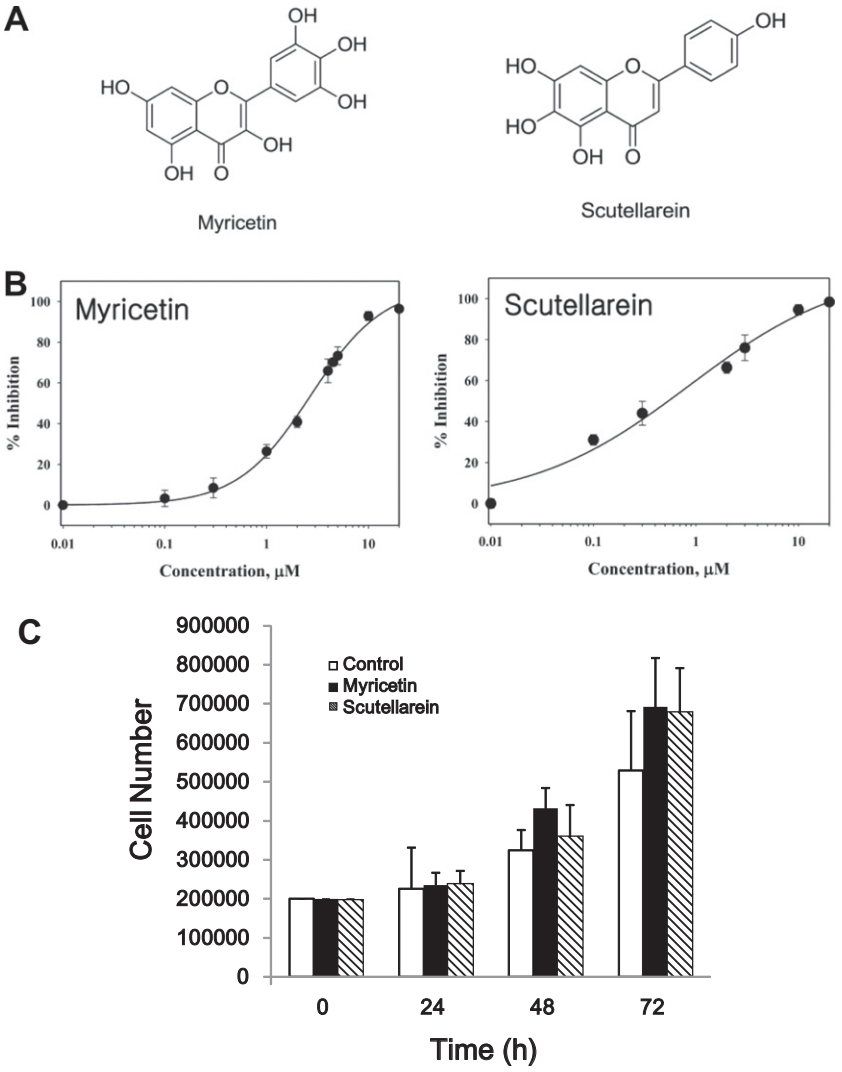

Figure 3. (A) Structure of myricetin and scutellarein. (B) IC_{50} value of nsP13 ATPase activity by myricetin and scutellarein. (C) The effects of myricetin and scutellarein on the growth of normal breast epithelial MCF10A cells.

mental setup. The reason for this discrepancy is currently unknown, but this may be due to structural difference of the ATPase domain between SARS-CoV helicase and HCV helicase. This result also indicates that suppression of SARS-CoV helicase by myricetin and scutellarein might not be mediated by affecting the protein stability and/or integrity of SARS-CoV protein in vitro, since these compounds did not seem to suppress the ATPase activity of HCV helicase protein. Therefore, it would be very interesting to examine which amino acid residues myricetin and scutellarein directly bind to on the SARS-CoV helicase to inhibit ATPase activity. Our modeling analysis shows that myricetin or scutellarein could fit in and directly interact with ATP/ADP binding pocket of the SARS-CoV helicase protein, thereby excluding a direct binding of ATP/ADP (Supplementary Fig. A). In particular, myricetin is likely to interfere with ATPase activity of the SARS-CoV helicase protein, possibly by directly interacting with critical residues of the ATPase domain, such as N265, Y269, and R443 (Supplementary Fig. B). Nonetheless, this structural proposition requires further experimental verifications in the future. Collectively, we propose that myricetin and scutellarein hold a great promise for use in treating and controlling potential future SARS outbreaks; however, more preclinical/clinical studies are necessary to examine whether this effect occurs after in vivo treatment.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.081. These data include MOL files and InChiKeys of the most important compounds described in this article.

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5. Natural compounds used in our study were directly purified from various medicinal plants or purchased from commercial vendor (Chromadex Inc.) (Table 1). The integrity of the individual natural compounds, directly purified from natural plants was confirmed by NMR spectroscopy (More specific information is available upon request.). All natural compounds were dissolved in DMSO at a concentration of 10 mM as a stock solution before experiments.
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7. SARS-CoV helicase, nsP13, was expressed in *Escherichia coli* Rosetta™ and purified. The helicase domain of HCV, NS3h, was overexpressed in *E. coli* BL21(DE3) and purified. TAMRA and fluorescein-labeled DNAs were purchased from Integrated DNA Technologies (Coralville, IA), and the concentrations were determined by absorbance at 260 nm and their extinction coefficients. The base sequences of TAMRA-labeled and fluorescein-labeled DNAs were as follows: 5′-20T25Tam (5′-T_{20}GAGCGGATTACTATACTACATTAGA(TAMRA)-3′), 3′-0T25Flu (5′-(Fluorescein)TCTAATGTAGTATAGTAATCCGCTC-3′), and 3′-15T25Flu (5′-(Fluorescein)TCTAATGTAGTATAGTAATCCGCTCT$_{15}$-3′), respectively. The SARS-CoV helicase substrate was prepared by annealing 5′-20T25Tam and 3′-0T25Flu and HCV NS3h substrate was prepared by annealing 5′-20T25Tam and 3′-15T25Flu. The 5′-20T25Tam and 3′-15T25Flu were designed to have a 20-base dT overhang at the 5′-terminus and a 15-base dT overhang at 3′-terminus to load the SARS-CoV helicase and HCV NS3h, respectively.
8. ATP hydrolysis by helicases was assayed by measuring the amount of released inorganic phosphate from ATP using a colorimetric assay. Colorimetric measurements of complex formation with malachite green and molybdate (AM/MG) were performed in the presence of various concentrations of natural compounds. All experiments were repeated three times and averaged.
9. Normal breast epithelial MCF10A cells were maintained in DMEM (Invitrogen, Carlsbad, CA) media, supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 0.02 μg/ml epidermal growth factor (EGF), 5 μg/ml insulin, 1.25 μg/ml hydrocortisone (Sigma, St. Louis, MO, USA) at 37 °C in 5% CO_2. MCF10A cells were seeded in six well plates at the number of 2.0×10^5 per well and exposed to myricetin or scutellarein at the concentration. Cells were collected every 24 h for 3 days and the viable cell number was calculated, using hemacytometer counting. Data are shown in mean ± standard deviation and a statistical analysis was conducted with Student *t*-test ($n = 6$). However, we did not observe any statistical significance between control group versus myricetin group or scutellarein group.
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