



Toona sinensis Roem tender leaf extract inhibits SARS coronavirus replication

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ABSTRACT

Aim of the study: Severe acute respiratory syndrome (SARS) is a life-threatening disease caused by the SARS coronavirus (SARS-CoV). The development of new antiviral agents for SARS-CoV is an important issue. We tried to find potential resource from Traditional Chinese medicine (TCM) for development of new drugs against SARS-CoV.

Materials and Methods: Our team recruited the potential TCM formulae (also known as Kampo) from two TCM books, Shang-Han Lun (Discussion of Cold-Induced Disorders) and Wen-Bing Tiao-Bein (Differential Management of Febrile Diseases). Several herbs, which were believed to be beneficial for SARS by experienced TCM doctors were also recruited. In addition, a vegetable popular in Taiwan, China and Malaysia, the tender leaf of *Toona sinensis* Roem (also known as *Cedrela sinensis*, belongs to the family Meliaceae) was also recruited under the suggestion of botanic experts. These TCM products and plant extracts were then tested for the effectiveness against SARS-CoV in vitro.

Results: Only TSL-1, the extract from tender leaf of *Toona sinensis* Roem was found to have an evident effect against SARS-CoV with selectivity index 12~17.

Conclusion: This paper reports for the first time that extract from a vegetable, the tender leaf of *Toona sinensis* Roem, can inhibit SARS-CoV in vitro. Therefore, the tender leaf of *Toona sinensis* Roem may be an important resource against SARS-CoV.

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1. Introduction

Severe acute respiratory syndrome (SARS) is a life-threatening disease caused by the SARS coronavirus (SARS-CoV) (Drosen et al., 2003). The overall mortality rate is around 10%. Therefore, the development of new antiviral agents for SARS-CoV is an important issue. In 2003, Cinatl et al. firstly reported the discovery of glycyrrhizin to inhibit replication of SARS-CoV, which suggested that

traditional herbs might be a potential resource for development of new drugs against SARS-CoV (Cinatl et al., 2003a).

A lot of herbs used in traditional Chinese medicine (TCM) may be of great treasure. The reason is TCM has been well organized and associated with thousands years of history in clinical practice. Among a lot of TCM books, two of those, named as Shang-Han Lun (Discussion of Cold-Induced Disorders) (Zhang, 220) and Wen-Bing Tiao-Bein (Differential Management of Febrile Diseases) (Wu, 1811), described some SARS like diseases and therapies. Therefore, our team recruited the potential TCM formulae (also known as Kampo) from these two books. Several herbs, which were believed to be beneficial for SARS by experienced TCM doctors were also recruited. In addition, a vegetable in Taiwan, China and Malaysia, the tender leaf of *Toona sinensis* Roem (also known as *Cedrela sinensis*, belongs to the family Meliaceae) (Fig. 1) was also recruited

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Fig. 1. Tender leaves of *Toona sinensis* Roem were shown as brown-red. Old leaves were shown as green.

under the suggestion of botanic experts. These TCM products and plant extracts were then tested for the effectiveness against SARS-CoV in vitro.

2. Materials and methods

2.1. TCM products and herbs

TCM formulae included Ger-Gan-Hwang-Lein-Hwang-Chin Tang, San-Hwang-Sei-Sin-Tang and Mar-Sing-Ther-Gang-Tang, Ger-Gan-Hwang-Lein-Hwang-Chin Tang, San-Hwang-Sei-Sin-Tang, Huang-Lein-Zhei-Du-Tang, Mar-Sing-Ther-Gang-Tang (the above developed around 196–219AC in Han dynasty), Pu-Zhi-Siau-Du-Yien (developed around 1180–1251 AC in Song dynasty), Yin-Chiau-San, Sang-Zhiu Yien (developed around 1800–1820 AC in Ching dynasty), Chu-Gen Tang, San-Hwang Sei-Sin-Ther-Gau-Yin (new developed). Herbs included Yin-Sing, Lei-Gong-Teng, Green tea, Hong-Jing Tein, Hwang-Lein and *Phyllanthus urinaria*.

S1, S2, S3 and S4 are four fractions from *Phyllanthus urinaria*, which has been used for treatment of hepatitis B. Green tea powder is produced by Ten Ren Tea Co., Ltd, Taipei, Taiwan. Green tea extract is produced by Chang Gung Biotechnology, Taipei, Taiwan. It contains 86.19% of EGCG and 12.13% of EGC.

2.2. *Toona sinensis* Roem and extracts

Toona sinensis Roem, also known as *Cedrela sinensis* A. Juss, is a famous plant in China. It has been cultivated for more than 2300 years. According to the “Tu-Zing-Bern-Tsau” (Atlas-Bible of Natural Herb) published in the Song dynasty around the 11th century, the leaf of *Toona sinensis* Roem had been described as “having sweet smell, and to be eatable”. It was introduced from Southern China to Taiwan in 1915. The tender leaf used in this experiment was collected from *Toona sinensis* Roem grown in Tuku, Yunlin County at the west side of Taiwan (Chang et al., 2002; Wang et al., 2008) (Fig. 2).

TSL-1 is a fraction of crude extract from the tender leaf of *Toona sinensis* Roem prepared according to previous report



Fig. 2. *Toona sinensis* Roem was grown in a farm at Tuku, Yunlin County, Taiwan.

(Wang et al., 2008). In brief, 100 g of tender leaves yield approximately 5–6 g of TSL-1 powder. Moreover, TSL-1 treated with nanometer-manufacturing technique (to make the particle less than nanometer) was designed as TSL-1nm. In the antiviral assay against SARS-CoV, they were dissolved in distilled water and then diluted in MEM supplemented with 2% FBS.

2.3. Antiviral screening test using HCoV 229E

The antiviral assays systems consisted of two systems. The first system was the screening test using HCoV 229E. It was performed at Taiwan as previously reported (Hsieh et al., 2004). In brief, MRC-5 cells, firstly treated with trypsin, and then were seeded onto 96-well plates with a concentration of $1 \times 100,000$ cells/ml and a volume of 70 μ l per well. After incubation at 37 °C with 5% CO₂ for 24 h, 20 μ l of HCoV (strain 229E) virus was added and incubated for another 2 h. 10 μ l of tested substances were then added to culture wells in triplicate in different concentrations. Actinomycin D was used as a positive control and 0.1% DMSO was used as negative control respectively. After incubation at 37 °C with 5% CO₂ for 4 days, the MTT test was carried out to determine the level of cell viability. The TCM products and extracts were tested at the concentration of 5 μ g/ml or 20 μ g/ml in the first study and at 50 μ g/ml and 200 μ g/ml in the second study.

2.4. Confirmation test using SARS-CoV

The antiviral activity against SARS-CoV strain FFM1 was done as previously described (Cinatl et al., 2003a). This assay was performed in a P3 laboratory. In brief, SARS-CoV was cultured in 96-well microplates on confluent layers of Vero cells. Cytopathogenicity induced by the SARS-CoV 72 h after infection was visually scored. Selectivity index (SI) was counted as the ratio of CC50 to EC50. CC50 denotes the concentration of the tested extract that reduced cell viability to 50%. EC 50 denotes the concentration of the tested extract needed to inhibit the cytopathic effect to 50% of the control value. The cytotoxicity of the drugs was determined with an MMT cell-proliferative Kit I (Roche, Mannheim, Germany).

2.4.1. Cell line

African green monkey kidney cell lines Vero (CCL-81) was obtained from ATCC (Manassas, VA, USA). Vero cells were grown at 37 °C in MEM supplemented with 10% fetal bovine serum (FBS) containing 100 IU/ml of penicillin and 100 μ g/ml of streptomycin.

SARS-CoV strain FFM 1 (Drosen et al., 2003) was prepared by infecting Vero cells cultures. Supernatants from infected cultures were collected 2 days post infection and aliquots were stored at -80°C . Virus titres were determined by 50% tissue culture infective dose (TCID₅₀) in confluent cells in 96-well microtiter plates as described (Cinatl et al., 2003a, 2005).

2.4.2. Antiviral assay

Confluent cell cultures were infected with SARS coronavirus strain FFM1 for 1 h in 96-well microplates. After adsorption period, cells were washed with PBS and incubated in MEM supplemented with 2% FBS. Cytopathogenic effect (CPE) was assessed visually 72 h after infection. Virus yield reduction assay was performed as described before (Cinatl et al., 2003b, 2005). Confluent cell layers grown in 12.5 cm² cell culture flasks were infected with SARS-CoV strain FFM 1. After 1 h incubation period, cells were washed four times with PBS and incubated (37°C) in MEM supplemented with 2% FBS. After 72 h cultures and supernatants were freeze-thawed and viral titres were determined by the 50% tissue culture infective dose in confluent Vero cells on 96-well microtiter plates. The inhibitory effects were expressed as effective concentrations of compounds required to inhibit infectious virus titres by 50% (EC₅₀).

2.4.3. Cell viability assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Mosmann, 1983) as described previously (Michaelis et al., 2004). Vero cells were seeded onto 96-well microtitre plates, grown to confluency, and incubated with culture medium containing TCM or herb extracts. Incubation time was analogous to that used for virus experiments. After incubation MTT (1 mg/ml) was added and after an additional 4 h cells were lysed in a buffer containing 20% (w/v) SDS and 50% *N,N*-dimethylformamide adjusted to pH 4.5. Absorbance at 570 nm was determined for each well using a 96-well multiscanner. After subtracting background absorbance, results are expressed as cell number compared to control cells that were maintained in the presence of solvent. CC₅₀ was determined as concentration that decreases cell viability by 50%.

3. Results

3.1. Antiviral effect against HCoV 229E of TCMs or single herb extract in the first study

In the first study against HCoV 229E, 12 potential TCM products and extracts were tested. They were three TCM formulae, Ger-Gan-Hwang-Lein-Hwang-Chin Tang, San-Hwang-Sei-Sin-Tang and Mar-Sing-Ther-Gang-Tang as well as nine herb extracts including Yin-Sing, Lei-Gong-Teng, Green tea powder, Green tea extract, S1, S2, S3, S4 and Hong-Jing Tein. All showed no antiviral activity at the concentration of either 5 ug/ml or 20 ug/ml (data not shown). Therefore, they were not further tested with SARS-CoV.

3.2. Antiviral effect against HCoV 229E of TCMs or single herb extract in the second study

In the second study against HCoV 229E, eight potential TCM products and extracts were tested. They were six TCM formulae including Ger-Gan-Hwang-Lein-Hwang-Chin Tang, San-Hwang-Sei-Sin-Tang, Mar-Sing-Ther-Gang-Tang, Yin-Chiau-San, Chu-Gen Tang, and San-Hwang Sei-Sin-Ther-Gau-Yin as well as two herb extracts from Hong-Jing-Tein and Hwang-Lein. Among these TCM

Table 1
Selectivity index against SARS-CoV of TCMs and *Toona sinensis* Roem extract

Number	TCMs or extracts	Form	CC50 (ug/ml)	EC50 (ug/ml)	SI*
1	Yin-Chiau-San	Boiled	>500 >500	>500 >500	>1 >1
2	Pu-Zhi-Siau-Du-Yien	Boiled	>500 >500	240 219	>2 >2
3	Ger-Gern-Hwang-Lein -Hwang-Chin-Tang	Boiled	>500 >500	134 280	>3 2
4	Sang-Zhiu-Yien	Boiled	>500 >500	349 326	>1 >1
5	Huang-Lein-Zhei-Du-Tang	Boiled	>500 >500	369 349	>1 >1
6	TSL-1	Boiled	>500 >500	30 43	17 >12
7	TSL-1nm	Boiled	>500 >500	37 70	>13 >7

*** Indicates selectivity index. ">" Indicates greater.

products and extracts, Yin-Chiau-San, Chu-Gen Tang, and San-Hwang Sei-Sin-Ther-Gau-Yin showed evident effect to inhibit viral replication at both 50 ug/ml and 200 ug/ml (76.23% and 143.18%; 99.13% and 116.52%; 56.23% and 143.18% individually). However, as tested with SARS-CoV, all were ineffective against SARS-CoV (data not shown).

3.3. Selectivity index against SARS-CoV of TCMs and *Toona sinensis* Roem extract

In the third study, five TCM formulae included Yin-Chiau-San, Pu-Zhi-Siau-Du-Yien, Ger-Gern-Hwang-Lein, Sang-Zhiu-Yien and Huang-Lein-Zhei-Du-Tang as well as *Toona sinensis* Roem tender leaf extract TSL-1 and TSL-1nm were tested against SARS-CoV. None of TCM formulae had evident effect against SARS-CoV. However, Both TSL-1 and TSL-1nm showed evident effect against SARS-CoV. The SI of TSL-1 was greater than 12 in regular condition and 17 after boiling of TSL-1. The SI of TSL-1nm was greater than 7 in regular condition and greater than 13 after boiling of TSL-1nm (Table 1). The mean SI was around 15 in TSL-1 and 10 in TSL-1nm.

4. Discussion and conclusion

Many potential agents against SARS-CoV in vitro have been identified (Cinatl et al., 2005). However, a major concern is the safety and experience in practical application in human beings. Much different from a lot of previously identified components or drugs against SARS-CoV, the tender leaf of *Toona sinensis* Roem has been used as a popular vegetable by Chinese people in both mainland China and Taiwan with high level of safety.

However, few studies were done regarding its scientific basis till the end of 20th century. In 2002, it has been reported that the crude extract of *Toona sinensis* Roem leaf can induce apoptosis of A549 lung cancer cells (Chang et al., 2002). The effect to improve lipolysis of differentiated 3T3-L1 adipocyte was also noted (Yang et al., 2003). In addition, the aqueous extract of *Toona sinensis* Roem can exert antiproliferative action and growth inhibition on HL-60 cells through apoptosis induction (Yang et al., 2006). Recently, *Toona sinensis* Roem leaf extract was proved to alleviate hyperglycemia via altering adipose glucose transporter 4 (Wang et al., 2008). However, the potential of *Toona sinensis* Roem for inhibition of SARS-CoV has not been reported so far. To our knowledge, this is the first report to show extract from the tender leaf of *Toona sinensis* Roem against SARS-CoV.

It is promising to find a new natural resource against SARS-CoV. However, the key compound in *Toona sinensis* Roem to inhibit SARS-CoV is still unclear. Recently, many compounds have been purified from the leaves of *Toona sinensis*. They include methyl gallate, gallic acid, kaempferol, quercetin, quercitrin, rutin, kaempferol-D-glucoside, (+)-catechin, (–)-epicatechin, beta-sitosterol, stigmasterol, beta-sitosteryl-glucoside, stigmasterol-glucoside, phytol and toosendanin (Chia, 2007). One of those compounds, quercetin, has been reported to have antiviral activity against HIV-luc/SARS, with a selective index of 40 (Yi et al., 2005). However, the key component in *Toona sinensis* inhibiting SARS-CoV still needs further investigations in the future.

In conclusion, this paper reports for the first time that extract from a vegetable, the tender leaf of *Toona sinensis* Roem, can inhibit SARS-CoV in vitro. Therefore, the tender leaf of *Toona sinensis* Roem may be an important resource against SARS-CoV.

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