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Full Length Research Paper

Antibacterial activities of commonly used traditional Chinese medicines as cold and flu remedies

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***Coptis chinensis*, *Andrographis paniculata*, *Isatis Radix*, *Lonicera japonica* Thunb, and *Senecionis Scandentis* are traditional Chinese medicine (TCM) widely used in China and eastern Asia for treatment of "warm diseases", including infectious diseases. They are main ingredients in many popular TCM formulas for "warm diseases". This study is to evaluate the antibacterial activity of *C. chinensis*, *A. paniculata*, *I. indigotica*, *L. japonica* Thunb, and *S. scandentis,* as well as the formulas containing these medicinal herbs. Those are commonly used medicinal plants from China used as cold, flu, and infectious diseases remedies. We have screened those medicinal herbs as well as five TCM formulas containing these herbs against four bacterial strains, *Staphylococcus aureus* (SA), *Bacillus atrophaeus* (BA), *Escherichia coli* O157:H7 (EC) and *Shigella dysenteriae* (SD). Our results showed that the tested medicinal plants and the TCM formulas have moderate antibacterial activity against SA, BA, SD and weak activity against EC. At higher concentration, they showed bactericidal activity. The findings support the use of the tested medicinal herbs and formulas to prevent and treat bacterial infections.**

Key words: Antibacterial activity, traditional Chinese medicine, infectious disease.

INTRODUCTION

Common colds, influenza and other acute infectious diseases are classified as "warm diseases" in traditional Chinese medicine (TCM). Many ancient formulas (based on herbal and natural products) for treating those acute infectious diseases have been developed through the history, and have been used for thousands of years. Their clinical benefits and efficacy as well as their safety have been demonstrated through its usage over the history, and yet, their mechanisms through modern scientific point of views have not been thoroughly examined. Common colds, influenza and other infectious diseases still belong to the widely spread diseases in today's world. While antibiotics have played critical roles in fighting bacterial infection in the last 80 years, no effective therapeutics and vaccines are available for common colds, a class of mild, but highly contagious diseases caused by rhinoviruses and coronaviruses. Influenza (also known as flu) is a life-threatening, highly contagious disease caused by influenza viruses (mainly by influenza viruses A and B and rarely by influenza virus C) (Centers for Disease Control and Prevention (CDC), 2010). The best way to prevent flu is through vaccinations (CDC, 2010). However, vaccine against flu needs to be modified or re-constructed every year due to rapid mutation rate of

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Abbreviations: **TCM**, traditional Chinese medicine; **CDC**, Centers for Disease Control and Prevention; **URIs**, upper respiratory tract infections; **OTC**, over the counter; **NB**, nutrition broth; **LB**, lubria broth; **UV**, ultraviolet; **NSRDEC**, Natick Soldier Research Development and Engineering Center; **ATCC**, American Type Culture Collection; **BA**, *Bacillus atrophaeus*; **SD**, *Shigella dysenteriae*; **EC**, *Escherichia coli* O157:H7; **SA**, *Staphylococcus aureus*; **LF**, *Lonicera Flower*; **IR**, *Isatidis Radix*; **AN**, *Andrographis Paniculata*; **RC**, *Rhizoma coptid*; **SS**, *Senecionis Scandentis*; **OD**, optical density; **TOA**, top overlay agarose; **MIC**, minimum inhibition concentration; **MDA**, micro-broth dilution assay; **SBM**, suitable bacterial-medium; **SFDA**, State Food and Drug Administration of PRC; **DMSO**, dimethylsulfoxide.

the flu viruses, and vaccination has to be done each every year. Despite this yearly vaccination efforts, there are incidences that the strains in the vaccine did not match the most prevalent strains circulating that season (CDC, 2008a, 2008b), or the new mutated strains emerging after the seasonal flu vaccine production (H1N1 flu outbreak in 2009), leading to low vaccine effectiveness (VE) for certain circulating strains during the flu season. Four antiviral drugs are approved so far by Food and Drug Administration (FDA) to treat flu: amantadine, rimantadine, zanamivir (Relenza®), and oseltamivir (Tamiflu®) (Chen et al., 2009; Roxas and Jurenka, 2007; Turner, 2009). The first two have been less effective since viral strains have adapted promising resistant against these drugs (Chen et al., 2009). Zanamivir and oseltamivir can reduce the period of illness if used within two days after the onset of symptoms (Chen et al., 2009; Roxas and Jurenka, 2007), but the resistant strains have been developed (CDC, 2009, Ujike et al., 2011) and some serious adverse effects affecting central nervous system and gastrointestinal-tract have been reported (Chen et al., 2009). Antibiotics are not helpful for the recovery of flu or common colds unless secondary bacterial infections have developed from the diseases (Turner, 2009). Despite of this, antibiotics are over-prescribed or even inappropriately prescribed for patients with cold, upper respiratory tract infections (URIs), or bronchitis (Gonzales et al, 1997; Roxas and Jurenka, 2007). The main reason of over-prescribed antibiotics, especially in children, may be due to patient pressure and/or parental pressure (Nyquist et al., 1998; Roxas and Jurenka, 2007). Unfortunately, widespread use of the antibiotics have in turn helped or forced bacterial strains to adapt and become resistant to the once-remarkable antibiotics, resulting in a global dilemma (Hedrick, 2006).

Every year in the U.S. from fall to spring, 5 to 20% of the population gets flu, more than 200,000 people are hospitalized from flu complications and about 36,000 people die from flu (CDC, 2010; Roxas and Jurenka, 2007). Although milder compared to flu, common colds account for more visits to the doctor than any other condition (American Lung Association (ALA), 2010a). Mostly diagnosed between September and May, adults average 2-4 colds per year, and children suffer 6-8 colds yearly (ALA, 2010b). There are no clinically-approved remedies for colds, though over-the-counter (OTC) cold medications may provide temporary relief of symptoms; an uncomplicated cold usually resolves in 10 days (Roxas and Jurenka, 2007). It is estimated that Americans spend more than $5 billion dollars annually on OTC drugs for colds and flu (Roxas and Jurenka, 2007; Associate Press, 2010). However, American Academy of Pediatrics has questioned the effectiveness of OTC cold medications for young children by addressing that the risk of their side effects may outweigh the benefits of relieving the symptoms, if any (Vernacchio et al., 2008).

Many TCM formulas for treating colds and flu have been used for more than 200 years (Wenbing Tiaobian, or Detailed Analysis of Epidemic Warm Diseases, first published in 1798). Based on TCM theory, the herbal medicines for treatment of the "warm diseases", such as infectious diseases (including colds and flu), are "cold and bitter" in nature. Through the modern extraction methods, many TCM formulas have been made into easy-to-use formulations, such as tablets, capsules, and granule powders, and are produced under good manufacture practice (GMP). Many of them have been collected into the Chinese Pharmacopeia, with the standardized manufacture procedures and identification/ analysis methods. Many TCM formulas of cold/flu remedies can be obtained as OTC without prescription in China, and can be obtained in US as supplements in Asian grocery stores. Some of the TCM herbal formulas are also adopted into the supplements for colds and/or flu in the mainstream pharmacy stores in US (that is, Airborne® cold remedy and related products, which contains *Lonicera Flower* (LF) and *Isatidis Radix* (IR)). A most recently study concludes that one TCM formula containing LF has similar effect of Oseltamivir (Tamiflu®) in the treatment of H1N1 influenza through a randomized clinical trial (Wang et al., 2011). While the effectiveness and safety of many TCM formulas for cold/flu have been demonstrated through hundreds of years of their consumption, vigorous scientific investigations of the raw herbs and formulations have not been carried out. In the present study, we have chosen five commonly used medicinal herbs and five formulas, both of which belong to "cold and bitter" class of TCM, to evaluate their anti-bacterial activity, as the first step to validate their usage in infectious diseases.

MATERIALS AND METHODS

All bio-tests and related materials were performed and handled, correspondingly, aseptically under Labcocon Class II Biosafety Cabinet (Labconcon Corp., Kansas City, MO). Prior to any bio-test/experiment, the working hood was sterilized with 70% ethanol and exposed to ultraviolet (UV) light for 20-30 min.

Preparation of nutritional mediums

All tests were performed using two nutritional mediums: nutrition broth (NB) and lubria broth (LB). For all the experiments, 1X NB and 1X LB medium were repeatedly prepared and used when needed. 1X NB medium contained roughly 8 g of NB powder (Sigma-Aldrich Co. St. Louis, MO) in 1 L of distilled water (that is, 8 g/L). 1X LB medium contained approximately 20 g of LB powder (Sigma-Aldrich Co. St. Louis, MO) in 1 L of distilled water (that is, 20 g/L). The mediums were then autoclaved for 20 min at 121℃ in a fluid cycle and stored at 4℃ upon cooling.

Preparation of nutritional agar plates

In order to make NB/LB-agar plates, 6 g of high gel strength agar

(Research Products International Co., Mt. Prospect, IL) was added to 1 L of the bacterial medium (NB or LB), and the resulting agar-medium solution was autoclaved. Immediately after autoclaving, molten NB/LB-agar solution was poured (roughly about 10-12 ml per each dish) in a number of sterilized petri-dishes. After the agar-dishes (or plates) had been solidified, they were directly used in the bioassays or stored upside-down at 4℃ for later use. In every experiment, the agar plates were strictly prepared under a sterilized-UV hood; all the sample mediums and agar-plates were only used and handled under the sterilized hood.

Preparation of bacterial cultures

Antibacterial properties of the TCM herbs were tested against four bacteria, *Staphylococcus aureus* (SA) (American Type Culture Collection (ATCC) 27217), *Escherichia coli* O157:H7 (EC) (ATCC 43888), *Bacillus atrophaeus* (BA) and *Shigella dysenteriae* (SD) (ATCC 13313). BA strain was from collections of US Army Natick Soldier Research Development and Engineering Center (NSRDEC). Strains of SA, EC and BA were given as a courtesy from NSDREC. A strain of SD was purchased as a lyophilized (freeze-dried) powder from ATCC (Manassas, VA), and was reconstructed into NB medium following ATCC's product instruction. LB medium was used for EC and BA, NB medium was used for SA and SD.

Prior to each bio-test, a fresh overnight bacterial culture was prepared and used in such experiments. Fresh overnight culture was prepared by adding 2 µl of the stock bacterial culture (stored at -80℃ and was thawed and brought to room temperature before use) into 2.0 ml of the appropriate medium. The liquid culture was incubated at 37℃ at 250 rpm for 8 to 10 h (reaching lo g phase).

Water extracts of raw herbs and determination of their antibacterial activity via spectrometric method

Five raw herbs were studied in this project as follow: LF*, Andrographis Paniculata* (AN)*, Rhizoma coptid (*RC*,* the dried rhizome of *Coptis chinensis),* IR, *Senecionis Scandentis* (Qian Li Guang, or SS). The extract powder form of the herbs LF, AN, RC, and IR were manufactured by Jiangyin Tianjiang Pharmaceutical Co. (Jiangsu, China). These herbal samples were vacuumed or sprayed dried from water decoction of the crude raw herbs. Two grams (2 g) of LF corresponded to 10 g of the LF raw herb; 1 g of AN corresponded to 10 g of its raw herb; 0.5 g of RC is approximately equivalent to 3 g of its raw herb; and 2 g of IR is approximately equal to 15 g of its raw herb. The SS extract powder was manufactured by KPC Products Inc. (Irvine, CA). One gram (1 g) of SS powder corresponded 5 g of raw herb.

Antibacterial properties of the five herbs were initially detected using spectrometric method (that is, by measuring optical density (OD) at 600 nm). To prepare for antibacterial tests, a stock solution in NB and LB medium for each of the raw herb was prepared as expressed in Table 1. Since the raw herbs were partially soluble in NB and LB medium, the solutions were refluxed in boiling water bath. As a result of the refluxing, all the herbs dissolved in both mediums. However, the solutions were noted highly (darkly) colored, making it difficult to detect the bacterial growth by the optical density measurements. Therefore, each of the solution was further diluted to make the detection of the bacterial growth manageable using OD measurement (see results and discussion section).

Ethanol extraction of the water-extracted herbs

About 2 to 5 g of each raw herb extract was dissolved in 200 ml of 100% ethanol and sonicated for 20 to 30 min. After filtering the undissolved material from the resulting solution, the ethanol from each herb solution was evaporated using a rotary evaporator. The herb residues were further dried under a high vacuum, weighed, and dissolved in distilled water. Since LF and SS extract did not fully dissolve in distilled water, dimethylsulfoxide (DMSO) was used for preparing extract stock for LF and SS.

Water and ethanol extraction of the herb formulas

Five herbal formulas studied in the current study are as follows: Yinhuang granules (Yinhuang), Shuanghuang Xiaoyan tablets (Shuanghuang), Banlangen granules (Isatis), Yinchiao tablets (Yinchiao), and Niuhuangshangqing tablets (Shangqing). Yinhuang granules contain two raw herbs: LF and *Scutellaria baicalensis*, manufactured by Yong An Pharmaceutical Co. Ltd (Yunnan, China). Shuanghuang Xiaoyan tablets, manufactured by Beijing Tong Ren Tang Traditional Chinese Medicine Co. (Beijing, China), contain *Berberis soulieana* and *S. baicalensis*. Banlangen granules (Isatis granules) were manufactured by Tianjin Zhongtian Pharmaceutical Ltd. (Tianjin, China). Isatis is made from the water extract of the raw herb *Radix isatidis* (aka indigowoad root). Yinchiao tablets and Niuhuangshangqing tablets are both manufactured by Tianjin Zhongxin Pharmaceutical Group Co. Ltd. (Tianjin, China). Yinchiao tablets consist of LF, *Forsythia*, *Mentha* herb, Chinese *Licorice* root, *Burdock* fruit, *Schizonepeta* herb, soybean seed, *Lophatherum* leaf, and balloon-flower root. Niuhuangshangqing tablets are a mixture of the extracts of the raw herbs including *C. chinensis* Franch, *S. baicalensis*, *Chrysanthemum* flower, *Forsythia*, and synthesized calculus Bovis (dried gallstones of cattle). Both Yinhuang and Isatis granules contains sugar in their formulations to improve the taste. Therefore, antimicrobial property of plain sugar was also tested. Yinhuang granules, Isatis granules, Yinchiao tablets, and Nuhuangshangqing tablets are listed in Chinese Pharmacopeia (2005 Edition), and manufactured based on Chinese Pharmacopeia standards. Shuanghuang tables are not listed in Chinese Pharmacopeia, and are manufactured based on the TCM Standards of The Ministry of Health of People's Republic of China. All five products are approved by State Food and Drug Administration of PRC (SFDA). Of the five formulas, only Yinhuang granule is completely soluble in water and medium. The rest are partially soluble in aqueous medium, therefore, we studied ethanol extraction for the other four formulas. About 2 to 15 g of the herb formulas (that is, Shuanghuang, Yinchiao, Isatis, and Shangqing) were weighted (in case of tablet, they were grinded into the powder using the Mortar and Pestles, before weighing), and were dissolved in 100% ethanol, followed by being sonicated for 20 to 30 min. After filtering, ethanol from each herbal extract was evaporated using a rotary evaporator. The dried herbal extracts were further dried under a high vacuum, weighed and dissolved in distilled water. Since the ethanol extract of Shuanghuang and Shangqing did not fully dissolve in distilled water, they were dissolved in DMSO as stock.

Antimicrobial activity screening of the raw herb and herb formulations against each bacterium using the top overlay agarose (TOA) method

The TOA method was performed as explained by Fankhauser (2010). Antimicrobial activity of each extract of the raw herbs was tested against each bacterium using the TOA method. On the freshly made NB/LB agar plates (as described above), 10 µl of 0.25 mg/ml Ampicillin (positive control), 10 µl of NB/LB medium depending on the bacteria being tested (negative control), 15 µl of the herbal extract of interest, and 15 µl of its corresponding solvent

Table 1. Listing of the concentrations of the prepared stock raw herbs and diluted solutions in the water based bacterial medium.

Raw herb sample	In NB medium		In LB medium	
	Stockconcentration (mg/ml)	Concentration of the diluted herb sample (mg/ml)	Stock concentration (mg/ml)	Concentration of the diluted herb sample (mg/ml)
LF/W	53.76	13.44	52.31	39.23
RC/W	12.97	5.1	7.626	3.050
IR/W	31.34	7.836	32.76	8.190
AN/W	12.84	2.568	11.12	2.223
Li/W	11.25	2.813	10.46	2.616

(DMSO or water) were spotted and allowed to completely dry. Ampicillin sodium salt was obtained from Sigma-Aldrich Co. (St. Louis, MO), and its solution was prepared using sterilized water, and filtered through 0.22 µm filter in the biological hood. The freshly grown overnight bacterial culture was obtained as discussed above. It was further diluted down 100-fold by adding 70 µl of the overnight culture in to 7.0 ml of the appropriate top agar, which is a molten agar-medium (NB or LB medium) at 45℃. Upon gently mixi ng, the mixed top agar was applied onto the spotted agar plates. After solidification of the top agar, each sample plate was incubated overnight at 37℃ for 10 to 12 h, and the zone of in hibition was measured for each spot using a ruler if noted.

Determination of the minimum inhibition concentration (MIC) range for the herb and formula extracts against selected bacteria using micro-broth dilution assay (MDA)

MIC range of each sample against each bacterium was determined using MDA (Black, 2005). In MDA, a 96-well microtiter plate was used to carry out serial dilution. Starting with various concentrations of the species, each was 2-fold serially diluted down the plate in triplicate. After obtaining each overnight bacterial culture, it (log phase) was diluted down 100-fold by adding 100 µL of the overnight culture in 10 ml of the suitable bacterial-medium (SBM). Next, 10 µl of the diluted bacteria culture was added to the test wells. All of the finalized wells had the volume of 110 µl. 10 µl of media were added to the NB and LB control wells. Bacterial controls did not have the herbal extracts. For certain controls, appropriate volume of DMSO is added. Next, the plate was wrapped using ethanol-wiped para-film and then re-wrapped with aluminum foil. The sample plates were incubated at 37℃ with a shaking speed of 150 rp m for 8 to 10 h. After incubation, the bacterial growth was monitored via measuring OD at 600 nm using a plate reader (SpectraMax M5, Sunnyvale, CA). Due to the dark color in some wells that was interfered with OD measurement, we did visual inspection to exam the bacterial growth in addition to OD measurements. Based on the visual observations (that is, turbidity) and OD measurements, the MIC of each species against each bacterium was reported. All of the experiments were repeated at least in two independent plates to obtain data-reproducibility. For ethanol extracts dissolved in DMSO, appropriate DMSO controls were included in the MDA to subtract the interference of DMSO, if any, on bacterial growth.

Determination of the bactericidal or bacteriostatic nature of the raw herbs and herb formulas

For each of the promising herb-bacterium pairs, sample herb solutions were prepared at the concentration close to but higher than their MIC in the SBM. There were two parts to the method. In part A (Run A), 100 µl of each sample solution was introduced to a 96-well microtiter plate in doublet. Each of the fresh bacterial culture was diluted down 100-fold by pipetting 70 µl of the fresh culture into 7 ml of its appropriate medium. 10 µl of the diluted bacterial culture was pipetted into the sample wells. In the control wells, 10 µl of the diluted bacterial culture was pipetted into 100 µl of the proper medium. Additional control wells were only treated with 100 µl of LB and NB medium. The sample plates were then incubated at 37℃ with a shaking speed of 150 rpm (or 250 rpm if the test is done in the test-tubes) for 6 to 10 h.

Next, part B (Run B) was performed. After the incubation period, from each of the sample wells, 10 µl of the sample solution was transferred into a new 96-well plate already containing 90 µl of the appropriate medium for the sample. It was made sure that the concentration of the sample in the new microtiter plate was less than its MIC. The new plates were incubated at 37℃ with a speed of 150 rpm for 8 to 10 h. The bacterial growth was noted by visual inspection of solution turbidity and via measuring OD of the new plates at 600 nm, and appropriate conclusions was determined.

RESULTS

Antibacterial properties of raw herb samples

Five raw herbs studied in this paper are all characterized as "cold and bitter" in nature, and used to treat "warm diseases", such as infectious diseases, based on the theory of TCM. Those raw herbal ready-to-use extracts have dark colors when suspended in water, which interfere with OD reading at 600 nm, therefore, they are diluted into the lower concentrations with water for the initial testing (Table 1). We chose four bacterial strains for the testing. SA and BA are gram positive bacteria, and EC and SD are gram negative bacteria. RC showed inhibition effect for SA, BA, and SD, with the MIC of 5.2 mg/ml, but did not show inhibition for EC at this concentration. LF showed inhibition effect for SD at 39 mg/ml. All the other raw herbs dissolved in water did not show bacterial inhibition. Since all the herbs suspended in water only showed weak or no antibacterial activities, we further performed ethanol extractions for these five raw herbs, and tested the antibacterial activities of ethanol extracts. The extraction yield was 2% for SS, 57% for RC, 16% for LF, and 9% for IR. The ethanol extract of each herb was first tested through TOA

Table 2. Antibacterial activity of the herb ethanol extracts (LI, RC, LF, AN, and IR) against each bacterium as determined via MDA.

Herb extract (solvent: water/W or DMSO/DM)	MIC of the herb extract (mg/ml)	Kill or inhibit (tested concentration, mg/ml)
Activity toward SA (G+)		
SS (W)	1.56	Inhibit
RC (W)	0.130	Kill (0.576)
LF (DM)	9.61	Kill (74.5)
AN (W)	21.9	Kill (76.1)
IR (W)	21.8	Kill (78.0)
Activity toward BA (G+)		
SS (DM)	14.3	Kill (44.4)
RC (W)	2.52	Kill (11.5)
LF (DM)	50.7	Kill (149)
AN (W)	37.4	Kill (66.0)
IR (W)	22.5	Kill (104)
Activity toward EC (G-)		
SS (DM)	> 30.1	Kill (101)
RC (W)	23.3	Kill (115)
LF (DM)	No activity observed	NA
AN (W)	46.0	Kill (101)
IR (W)	45.7	Kill (104)
Activity toward SD (G-)		
SS (DM)	30.1	Kill (30.1)
RC (W)	5.29	Kill (14.4)
LF (DM)	20.2	Kill (74.5)
AN (W)	46.0	Kill (101)
IR (W)	45.7	Kill (104)

*: W, indicating the ethanol extract stock solution was prepared in water; DM, indicating the ethanol extract stock solution was prepared in DMSO. Note: G+/-, gram positive/negative.

method, followed by the MDA to determine the MIC. The results are listed in Table 2. Ethanol extract stock solutions were prepared either in water, or in DMSO (as listed in Table 2), depending on their solubility. When DMSO was used as solvent for stock solution, the appropriate DMSO was used as controls during assay.

RC ethanol extract showed the strongest antibacterial activity for all four strains of bacteria. It showed low single digit mg/ml MIC for three bacterial strains, SA (0.13 mg/ml), BA (2.5 mg/ml), and SD (5.3 mg/ml). For EC, RC ethanol-extract showed fair inhibition with MIC of 23 mg/ml. One of the major components of RC is berberine (WHO, 1999), which has been previously reported having promising antibacterial activity (Yu et al., 2005). Our preliminary data also showed promising antibacterial activity of berberine against SA (MIC: 52 µg/ml), BA (MIC: 109 µg/ml), SD (MIC: 207 µg/ml), but with very poor activity against EC (MIC>2 mg/ml) (Shah, 2011). All the results considered together suggested that there might be other components in herb RC, which contributed to its antibacterial activity against EC. RC has been widely used in TCM to treat dysentery and other bacterial enteritis, as well as other infectious related diseases, including URI. RC is also used in TCM to treat other "warm diseases".

LF ethanol extract showed good inhibition for SA (MIC: 9.6 mg/ml), but only weak inhibition on SD (MIC: 20 mg/ml), and BA (MIC: 51 mg/ml). LF ethanol extract did not show observable inhibition against EC (Table 2). IR and AN ethanol extract showed weak inhibition against SA (MIC: 22 mg/ml for both), BA (MIC: 23 mg/ml for IR, and 37 mg/ml for *AN*), EC (MIC: 46 mg/ml for both), and SD (MIC: 46 mg/ml) (Table 2). LF and IR have been used in TCM for treating and preventing infectious diseases, such as cold, flu, URI, some skin infections (through topical and oral uses), as well as other "warm diseases".

AN is used for treating URI and dysentery in TCM. SS extract showed inhibition against SA (MIC of 1.6 mg/ml), BA (MIC of 14 mg/ml) and SD (MIC of 30 mg/ml), but poor inhibition against EC (>30 mg/ml) (Table 2). To improve the solubility of SS ethanol extract, DMSO was used to prepare the stock SS solution for assay against BA, EC, and SD (Table 2). SS is a main component of one proprietary TCM formula approved by SFDA as OTC for treating of sinusitis in China for decades. However, a recent report found that SS contains hepatotoxic natural toxin pyrrolizidine alkaloids in the raw herb, which has not been detectable in the OTC formula (Li et al., 2008). Nonetheless, usage of high dosage of SS should be careful.

Further, we determined the bactericidal or bacteriostatic nature for those selected TCM. RC water extract showed bactericidal properties at 10 mg/ml against SA. For ethanol extract, RC, LF, AN, and IR showed bactericidal activity against SA, BA, and SD at higher concentration (Table 2), SS showed bactericidal activity against BA and SD at higher concentration (Table 2), SS, RC, AN, and IR showed bactericidal activity against EC at higher concentration (Table 2).

Antibacterial properties of herb formulas

The above raw herbs have been widely used in different formulas for treating and preventing "warm diseases", including infectious diseases. Most of the time, however, they are used in a formula in combination with each other and/or other TCMs. The combinations of herbs have been formulated into the easy-to-use formulations, such as granules, tablets, and capsules. In our study, we chose five formulas that are approved by SFDA as OTC to treat cold and URI related diseases. Two of these formulations are in granule formulation (Yinhuang and Isatis), and the other three are in tablet formulation (Shuanghuang, Yinchiao, and Shangqing). Yinhuang granules can be completely dissolved into water and in the nutritional medium. The rest of the formulas were only partially dissolved into the aqueous medium.

Yinhuang aqueous solution showed inhibition against SA (MIC of 16 mg/ml) and SD (MIC of 42 mg/ml), but did not show inhibition against EC and BA. Shuanghuang showed inhibition activity against SA and BA at 15 mg/ml, but did not show inhibition activity against EC and SD. The other three formulas dissolved in aqueous medium depicted weak to no inhibition activity against all bacterial strains tested. Furthermore, Yinhuang showed bactericidal activity against SA at 40 mg/ml, and against SD at 100 mg/ml. Since both Yinhuang and Isatis granules contain sugar in their formulations, we tested the inhibition effect of sugar on bacteria growth, and showed no inhibition effects at 150 mg/ml, which is above the Yinhuang's MIC. Thus, we concluded that sugar did not play any role in the antibacterial activity of Yinhuang granules.

To further characterize the antibacterial activities of the formulations partially dissolved in the aqueous medium, we carried out ethanol extract for Shuanghuang, Yinchiao, Isatis, and Shangqing. The extraction yield is 11% for Shuanghuang, 2% for Isatis, 4% for Yinchiao and 5% for Shangqing. Similar to raw herbal extract, the ethanol extract of each formula was first tested through TOA method, followed by the MDA to determine the MIC for each extract. Depending on the solubility, again, either water or DMSO was used as solvent to prepare stock solution of the ethanol extract (Table 3).

Shuanghuang showed inhibition against SA (MIC of 1.5 mg/ml), BA (5.6 mg/ml), EC (52 mg/ml), and SD (13 mg/ml) (Table 3). Shuanghuang showed bacteriostatic activity, but showed bactericidal activity for EC at 183 mg/ml, and SD at 23 mg/ml. Isatis ethanol extract showed inhibition activities against SA (MIC of 15 mg/ml), BA (MIC of 22 mg/ml), EC (31 mg/ml) and SD (31 mg/ml). Higher concentration of Isatis ethanol extract showed bactericidal activity against SA (51 mg/ml), BA (102 mg/ml), EC (102 mg/ml), and SD (102 mg/ml). For Yinchiao ethanol extract, inhibitions against SA, BA, and SD were observed, with MIC of 11 mg/ml, 37 mg/ml, and 11 mg/ml, respectively. Yinchiao ethanol extract also showed bactericidal activities at higher concentration (50 mg/ml for SA, 99 mg/ml for BA, and 74 mg/ml for SD). However, Yinchiao ethanol extract did not show inhibition effect on EC. Shangqing ethanol extract showed inhibition against SA (MIC of 2.8 mg/ml), BA (MIC of 45 mg/ml), EC (MIC of 11 mg/ml), and SD (MIC of 24 mg/ml). Higher concentration of Shangqing extract showed bactericidal effect against SA, BA, EC, and SD, at the concentration of 6, 75, 46 and 24 mg/ml, respectively.

DISCUSSION

All the selected raw herbs belong to "cold and bitter" class of TCM used to treat and prevent "warm diseases" based on TCM theory. These herbs have been widely used to treat and prevent infectious diseases for hundreds years in China. However, their mechanisms of action are still not well understood. It has been hypothesized that those "cold and bitter" class TCMs have the antimicrobial activities, as well as immune boosting effects, but no thorough research has been carried out to prove those hypotheses. While many claims have been made about their antibacterial activities, few papers have been published in peer-reviewed journals, especially journals in the western world. This is primarily because those herbs are mainly used in Asian countries, and associated with so called “folk medicine”, without much scientific driven research, thus is neglected by mainstream western researchers. In

Table 3. Antibacterial activity of the formulation extracts (Shuanghuang, Isatis, Yinchiao, and Shangqing) against each bacterium as determined via MDA.

Herb extract (solvent: Water/W or DMSO/DM)	MIC of the herb extract (mg/ml)	Kill or inhibit (tested conc.,mg/ml)
Activity toward *S*A (G+)		
Shuanghuang (DM)	1.52	Inhibit
Isatis (W)	14.7	Kill (50.9)
Yinchiao (W)	11.1	Kill (49.6)
Shangqing (DM)	2.81	Kill (5.78)
Activity toward *BA* (G+)		
Shuanghunag (DM)	5.60	Inhibit
Isatis (W)	22.0	Kill (102)
Yinchiao (DM)	37.2	Kill (99.2)
Shangqing (W)	45.0	Kill (74.5)
Activity toward *EC* (G-)		
Shuanghuang (DM)	51.9	Kill (183)
Isatis (W)	30.8	Kill (102)
Yinchiao (W)	No observed activity	NA
Shangqing (DM)	11.2	Kill (46.2)
Activity toward *SD* (G-)		
Shuanghuang (DM)	12.8	Kill (22.9)
Isatis (W)	30.8	Kill (102)
Yinchiao (W)	11.1	Kill (74.4)
Shangqing (DM)	23.56	Kill (23.1)

*: W, indicating the ethanol extract stock solution was prepared in water; DM, indicating the ethanol extract stock solution was prepared in DMSO. Note: G+/-, gram positive/negative.

this paper, we, for the first time, report their antibacterial activities against several bacteria clinically relevant to human infectious diseases. Both SA and SD are pathogenic strains, EC is nonpathogenic strain of O157:H7, with the deletion of the pathogenic gene responsible for Shiga-like toxin I or II, and is used as a surrogate to study the pathogenic strain of EC (Gregory and Mello, 2005). BA is a non-pathogenic strain, but used as a surrogate of *Bacillus anthracis*, which is responsible for causing anthrax (Burke et al., 2004; Plomp et al., 2005). Our observations and findings support the use of these herbs for infectious diseases. Traditional use of TCM is through boiling into a decoction. To modernizing TCM, many TCMs have been decocted, concentrated and dried as powder forms, which are ready to dissolve into water for easy use. In this paper, we tested the decocted dry powder forms of the raw herbs since more and more TCM practitioners now use them for the convenience.

While the tested raw herbs studied can be used as a single herb in some cases, most of the time, they are combined each other, and/or with other TCMs into the specific formulas, which are believed to have synergetic effect among different TCMs, and thus better efficacy. Many of those formulas have been collected and used for hundreds of years (Wu, 1798). In this study, we have chosen five formulas used to treat "warm diseases" Yinhuang is made from two TCM herbs, LF and *S. baicalensis.* There are reports that the baicalin, a main alkaloid found in *S. baicalensis* have antibacterial activity (Liu et al., 2000), but our results, as well as other reports, showed that baicalin only showed weak antibacterial activity (WHO, 2007; Shah, 2011). Those conflict reports might be due to different bacterial strains used in different studies, but at the least, the studies indicate that baicalin, by itself, might not be a strong antibacterial agent. LF has also been speculated with antibacterial activity. Our results suggested that the main antibacterial activity might come from *Lonicera,* and that *S. baicalensis* may only play an accessory role. For the first time with this study, the promising antibacterial activity of Yinhuang formula has evidently been confirmed. Yinhuang formula has been used in China for colds, flu, URI, and other infectious diseases. Our findings support those indications for bacterial infections. Shuanghuang tablets contains *B. soulieana* and *S. baicalensis*. *B. soulieana* contains berberine, which might be responsible for the observed antibacterial activity of Shuanghuang, and

S. baicalensis could serve accessory role in this formula. Isatis granule is the single herb formulation of isatis root.

Our results showed comparable inhibition activities of Isatis root (raw herb IR) and Isatis formula against the tested bacteria. This observation supports the use of Istas root for treating infectious diseases. Yinchiao formula has long usage history for treating cold, flu, and other "warm disease" (it has been collected in Wenbing Tiaobian, or Detailed Analysis of Epidemic Warm Diseases, more than 200 hundred years ago). It is consisted of LF, *Forsythia*, *Mentha* herb, Chinese *Licorice* root, *Burdock* fruit, *Schizonepeta* herb, soybean seed, *Lophatherum* leaf, and balloon-flower root. LF and *Forsythia* are believed to have antibacterial activity. Our results showed antibacterial activities of Yinchiao tablets against SA, as well as BA and SD. Their inhibition activities are better than LF raw herbal powder, suggesting the combination formula might have better efficacy than *Lonicera* itself. The observations support the uses of Yinchiao for URI and other bacterial infections. Shangqing tablets are a mixture of the extracts of the raw herbs including *C. chinensis* Franch, *S. baicalensis*, *Chrysanthemum* flower, *Forsythia*, and synthesized calculus Bovis (dried gallstones of cattle). *C. chinensis* Franch and *Forsythia* might contribute the observed antibacterial activities. Shangqing have been used for a range of "warm diseases", including URI, infection of gums, as well as constipation. In TCM theory, constipation is related to "warm diseases", as the result of the accumulation of toxic substances due to constipation. Our results support the use of Shangqing for bacterial infections.

The observed antibacterial activities in our findings are relatively moderate for both the raw herbs and formulas. Their effectiveness through the history would suggest that in addition to their moderate antibacterial activities, other mechanisms may also be involved. Further purification and separation of the crude herb extract would help to find more potent antibacterial compound(s) and better characterize the herbs.

In our preliminary studies, we had fractionated the ethanol extract of *Lonicera* flower using column and thin-layer chromatography and found MIC in low µg/ml of certain fractions against *SA* (Shah, 2011). Although potent antibacterial extract(s) or compound(s) from TCMs could be effective *in vitro*, it is critical to note that the efficacy of TCM formulas, as the hypothesis of TCM states, is based on synergism between multiple TCMs in the formula and their overall interactions to the whole human body rather than with any specific affected parts(s) of the body. The moderate antibacterial activities of all the tested samples in this report provide pharmacological basis for their desired indications. Moreover, the common dosage requirements of raw herbs as remedies for common colds, flu, or other infectious diseases lay in the range of 15 to 60 grams per day (boiling into a decoction, the ready-to-use formulations have the equivalent dose). Noted effectiveness of such high dose use of the "cold and bitter" class of TCM may, in turn, be to compensate for their moderate antibacterial activity. There has been a very good safety profile of such high dose used for these medicinal plants through the history. Therefore, even though their antibacterial activities are moderate, TCMs provide important alternatives, and/or complementary therapies to antibiotics for infectious diseases, especially for chronic and difficult-to-treat conditions, and may help reduce the use of antibiotics, or fight with booming antibiotics-resistant bacterial strains.

Only a few antibacterial medicinal plants have been studied for their mechanisms of action. This is mainly due to the fact that not too many active components are known for those medicinal plants. Of those few examples, berberine, the active antibacterial component of RC and other berberine containing herbs, is the mostly studied. Berberine has shown antibacterial activity, especially to gram positive bacteria (Wang et al., 2009; Yu et al., 2005; Tegos et al., 2002), and it is believed that the antibacterial activity comes from its quaternary ammonium structure (Wang et al., 2009).

While finding the active component(s) in medicinal plants will play critical role in the medicinal plant research, it is worth to note that the action of many medicinal plants might be the results of their synergistic effects through multiple components, and only focusing on searching of single active component might overlook their overall action of mechanism. In this study, we choose the whole herbs and their formulas as the study subject, and we not only identify their minimum inhibition concentrations against several clinical relevant bacterial (both gram positive and negative), but also observed that most of them exhibit bactericidal activity at higher concentrations, including formulas we tested. The advantages of TCM are their proved effectiveness and safety through the history. However, not many basic and clinical investigations have been carried out in an attempt to find the mechanisms of TCM.

We report the first systematic study of antibacterial activities of a range of "cold and bitter" class of TCM and their formulas. Further study of antiviral activities of this class of TCM is also needed to support their claim for treatment of virus infections, such as colds and flu. In addition to pharmacological studies, the rigid and well-designed clinical studies are needed to find their true efficacy. Further research on these "cold and bitter" class of TCM may lead to better understanding of their mechanism and function as the remedies for infectious diseases, including colds and flu, and provide evidence-based bases to use them for treatment of infectious diseases.

Conclusions

Our results showed that all five raw herbs in the "cold and

bitter" class of TCM used for treatment of "warm diseases", including common colds and flu, have moderate antibacterial activity. The formulas containing these raw herbs also showed moderate antibacterial activity. The findings provide pharmacological evidence on traditional use of this class of TCM for treatment of infectious diseases.

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