

Full Length Research Paper

Screening of antibacterial activity of 20 Chinese herbal medicines in Yunnan

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Accepted 17 June, 2013

The 80% ethanol extracts from 20 Chinese herbal medicines collected in Yunnan Province, China, were screened for antibacterial activity against *Staphylococcus aureus* (SA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (EC) and *Pseudomonas aeruginosa* (PA), including antifungal activity against *Candida albicans* (CA) by the agar hole diffusion test. The extracts were prepared through macerating at room temperature and hot refluxing procedures. 19 of the 20 extracts showed effects with different potency and their inhibition zone diameters (IZD) ranged from 9 to 27 mm. The extracts from *Psychotria henryi* Levl and *Marsdenia tinctoria* R. Br. (maceration and reflux extraction) were the most active against both SA and MRSA, with mean IZDs = 19.7 to 22.0 mm. The anti-SA and MRSA active extracts were further determined for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by serial dilution with a standard broth microdilution method. 12 extracts showed effects on both SA and MRSA strains with MICs/MBCs ranges among 32 to ≥ 1024 / 64 to ≥ 1024 mg/L, respectively. *P. henryi*, *M. tinctoria* and *Sapium baccatum* Roxb also showed inhibition against EC, PA and CA strains, and displayed a broad spectrum of antimicrobial activities.

Key words: Chinese herbal, methicillin-resistant *Staphylococcus aureus* (MRSA), minimum inhibitory concentrations (MIC).

INTRODUCTION

Since methicillin-resistant *Staphylococcus aureus* (MRSA) occurred in early 1961 (Jevons, 1961), it has become a troublesome pathogen responsible for serious infections owing to its resistance to a large group of antibacterial agents, resulting in high morbidity and mortality. It is resistant not only to β -lactam antibiotics, but also to other antibacterial groups, such as fluoroquinolones, tetracyclines, macrolides, lincosamides and aminoglycosides. Some strains that are partially or fully resistant to vancomycin have been found (Pantosti and Venditti, 2009). The search for novel drugs to combat

MRSA infection is urgently needed.

Traditional Chinese medicine (TCM) has accounted for a wealth of health needs over thousands of years in China (The Compiler Group, 2009). A great number of plant species have been used as healing herbs in TCM for the treatment of infectious diseases, such as skin ulcers, cold, diarrhea, pneumonitis and tuberculosis, etc (Jiangsu New Medical College, 1977; Sima et al., 2012; Jinous and Fereshteh, 2012; Muhammad et al., 2012). There is increasing interest in researching Chinese herbal drugs for application as antibacterial agents (Zuo et al.,

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Table 1. The antibacterial agent-susceptibility testing results of MRSA strains.

MRSA Strain	Resistant (R)	Intermediate (I)	Susceptible (S)
MRSA092	PEN,OXS,AMP,AZM,CFZ,CAT,FUR,LEV	None	VAN,GAT,PIP/T,FOS,LNZ
MRSA098	PEN,OXS,CFP/SU,LEV,CLI,AZM,	GM	VAN,FOS,LNZ
MRSA111	PEN,OXS,FUR,LEV,ERY,PIP/T,CTX V	None	VAN,LNZ

AMP: ampicillin; AZM: azithromycin; CAT: cefathiamidine; CFP/SU: cefoperazone /sulbactam; CFZ: cefazolin; CLI: clindamycin; CTX: Cefotaxime; ERY: erythromycin; FOS: fosfomicin; FUR: cefuroxime; GAT: gatifloxacin; GM: gentamicin; LEV: levofloxacin; LNZ: linezolid; OXS: oxacillin; PEN: Penicillin; PIP/T: piperacillin/tazobactam; CFP/SU: cefoperazone /sulbactam; VAN: Vancomycin.

2008). For the sake of finding more anti-MRSA plants, we herein report the screening of 20 southern Yunnan medicinal plants for *in vitro* inhibition against clinical MRSA isolates, including their effects on pathogens of *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*.

MATERIALS AND METHODS

Bacterial strains

Strains of *S. aureus* (SA, ATCC25923), *E. coli* (EC, ATCC25922), *P. aeruginosa* (PA, ATCC27853), *C. albicans* (CA, ATCCY0109) and antibiotic susceptibility disks were provided by the National Institute of Control of Pharmaceutical and Biological products (NICPBP, Beijing, China). MRSA strains were clinical isolates from infectious samples of critically ill patients in Kunming General Hospital (KGH) (Table 1). Pathogen purification and identification (including colonial morphology, gram staining and coagulase testing) were conducted in our clinical microbiology laboratory and further confirmed by standard cefoxitin disk diffusion test following Clinical and Laboratory Standards Institute (CLSI, 2007) standard procedures. The tested MRSA strains were multi-drug resistant to β -lactams, aminoglycosides, fluoroquinolones and macrolides, and they were susceptible to vancomycin (Table 2).

Plants

The selected plants were collected in Xishuangbanna of Yunnan Province in August, 2011. They were identified at the Botany Department, Kunming Institute of Botany (KIB), the Chinese Academy of Sciences. The voucher specimens were preserved at the herbarium of KIB (Table 2).

Media used

Standard Mueller-Hinton agar and broth (MH-A and MH-B), and Sabouraud agar and broth (S-A and S-B) (Tianhe Microbial Agents Co., Hangzhou, China) were used as the bacterial and fungal culture media, respectively.

Extract preparation

Two types of 80% ethanol extracts were prepared from the 20

Southern Yunnan plants by the macerating extraction at room temperature (25°C) and the hot reflux extraction (85°C), respectively as the follows: An amount (50 g, each) of powdered air-dried aerial parts of the plant material was macerated with 80% ethanol (150 ml) for one week, filtered and the residue was further macerated twice with the same amount of solvent overnight and filtered after sonication for 30 min. The filtrates were combined and the solvent was evaporated at 40°C under reduced pressure to afford each of the plant extracts. Another amount (50 g, each) of the same plant material was refluxed with 80% ethanol for two hours, filtered and the residue was further refluxed twice with the same amount of solvent and filtered. The filtrates were combined and the solvent was evaporated at 40°C in vacuum to afford each of the plant extracts (Table 3).

Susceptibility test

The ethanol extracts of the 20 plants were initially subjected to susceptibility test according to the agar diffusion method on MH-A (for the bacteria) or S-A (for *C. albicans*) plates (Zuo et al., 2008). Briefly, crude extracts were prepared at concentrations of 50 mg/ml in dimethylsulfoxide (DMSO). The microbial suspension was firstly plated onto the agar plates with inoculums of 1.5×10^8 CFU/ml for the bacteria and 5×10^5 CFU/ml for *C. albicans*. Then, holes of 6.0 mm diameter each in the agar plates were punched with a sterilized hole-puncher. The samples were pipetted into the holes with no overflowing. The plates were incubated at 35°C for 24 h and measured and the inhibition zone diameters (IZDs) recorded. For every experiment, a sterility check (DMSO and medium), negative control (DMSO, medium and inoculums) and positive antibacterial control (vancomycin) were included (Table 3). All experiments were performed in triplicate.

The samples of IZDs > 12 mm against standard *S. aureus* (SA) were subjected to assay of their IZDs against MRSA (Table 4). The subsequent samples with IZDs > 12 mm against one of the MRSA strain (MRSA111) were further subjected to assay of their minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for MRSA by standardized broth microdilution techniques (Zuo et al., 2008). Briefly, the starting inoculums were 5×10^5 CFU/ml. The 96-well plates were incubated at 35°C for 24 h according to CLSI guidelines (CLSI, 2006, 2007). All experiments were determined in duplicate, with concentrations ranging up to 2048 mg/L. For the MBC assays, 0.1 ml aliquots from drug dilution wells with no visual growth inhibition were plated onto MH-A. The lowest drug concentration that yielded three or fewer microorganism colonies was recorded as the MBC (NCCLS, 1999). Vancomycin (Eli Lilly Japan K.K., Seishin Laboratories) was also used as a positive control anti-MRSA agent (Table 5).

Table 2. Species of the 20 medicinal plants.

No	Species	Family	Specimen number	Folk use
1	<i>Aphanamixis grandifolia</i> Bl.	Meliaceae	HITBC7568	Rheumatism and analgesia
2	<i>Blumea densiflora</i> DC.	Compositae	KUN3413	Rheumatism and analgesia
3	<i>Cinnamomum bejolghota</i> (Buch.-Ham.) Sweet	Lauraceae	KUN48	Ulcer, dysmenorrheal, rheumatism and ostalgia, injuries from falls
4	<i>Citrus medica</i> L.	Rutaceae	HITBC5184	Dyspepsia, cough
5	<i>Croton tiglium</i> L.	Euphorbiaceae	KUN51	Expelling phlegm, anthelminthic
6	<i>Duranta repens</i> L.	Verbenaceae	KUN662	Malaria, subduing swelling and detoxicating
7	<i>Fructus piperis</i> Nigri	Piperaceae	KUN5478	Dissolving phlegm and detoxicating, dyspepsia, diarrhea, epigastralgia
8	<i>Hedyotis capitellata</i> Wall. ex G. Don	Rubiaceae	KUN1217	Cold and rheumatism, malaria, fractures
9	<i>Heracleum candicans</i> Wall. ex DC	Umbelliferae	KUN12451	Asthma, cough and cold, chronic bronchitis, stomach ache, rheumatalgia, injury from fall
10	<i>Illicium simonsii</i> Maxim.	Magnoliaceae	IBSC125	Ulcer, anthelminthic
11	<i>Lignum santali</i> Albi	Santalaceae	HITBC5636	pneumonitis, cough and asthma, nausea and vomiting, ecchymoma, paralysis
12	<i>Marsdenia tinctoria</i> R. Br.	Asclepiadaceae	KUN722	Epigastralgia, hepatomegaly, rheumatism and osteodynia
13	<i>Millettia pachycarpa</i> Benth.	Leguminosae	YFS1005	Injury from fall, fracture, acute gastroenteritis
14	<i>Nerium indicum</i> Mill.	Apocynaceae	HITBC383	Cardiotonic and diuresis, expelling phlegm, analgesia, removing blood stasis, epilepsy
15	<i>Psychotria henryi</i> Levl	Rubiaceae	KUN6642	Strengthening spleen and dehumidify, regulating vital energy and analgesia
16	<i>Radix curcumae</i> Wenyujin	Zingiberaceae	KUN753	Menoxenia, jaundice, hemuresis, epilepsy
17	<i>Rumulus uncariae</i> Macrophyllae cum Uncis	Rubiaceae	KUN14506	Headache, serious dysentery, convulsion and hypertension
18	<i>Sapium baccatum</i> Roxb	Euphorbiaceae	KUN270	Menoxenia, dyspepsia, pa-leng
19	<i>Scutellaria discolor</i> Wall. ex Benth.	Lamiaceae	KUN688	Tympanitis, diminish inflammation, rheum, fever, tuberculosis, injury from fall
20	<i>Toona ciliata</i> Roem.var.pubescens (Franch.) Hand.-Mazz.	Meliaceae	PE53414	Tinea, chronic diarrhea, chronic dysentery, uterine bleeding

RESULTS

The antibacterial agent susceptibility spectrum of the tested three MRSA strains is listed in Table 1. They were used for anti-MRSA evaluation. The 20

Yunnan medicinal plants are shown in Table 2, together with their plant families, specimen numbers and folk uses. The initial antimicrobial screening results (IZDs) of 50 mg/ml ethanol extracts of the 20 plant extracts against standard

SA, that is, methicillin-susceptible *Staphylococcus aureus* (MSSA, ATCC25923) and other standard strains of EC, PA and CA are shown in Table 3. 19 of the 20 extracts showed effects with different potency and their IZDs ranged from 9 to 27 mm.

Table 3. Antimicrobial activities of 20 plant ethanol extracts (IZD unit: mm).

Species	Extraction yield (%)	SA	EC	PA	CA
<i>A. grandifolia</i> (macerating extraction)	6.68	0	0	0	0
<i>A. grandifolia</i> (reflux extraction)	6.53	9	0	0	0
<i>B. densiflora</i> (macerating extraction)	5.51	0	0	0	0
<i>B. densiflora</i> DC. (reflux extraction)	3.02	0	0	0	0
<i>C. bejolghota</i> (macerating extraction)	6.20	0	0	0	20
<i>C. bejolghota</i> (reflux extraction)	7.68	16	0	0	10
<i>C. medica</i> (macerating extraction)	9.95	0	0	0	0
<i>C. medica</i> (reflux extraction)	41.42	0	0	0	0
<i>C. tigilium</i> (macerating extraction)	4.31	12	0	0	0
<i>C. tigilium</i> (reflux extraction)	9.18	13	0	0	14
<i>D. repens</i> (macerating extraction)	7.00	11	0	0	0
<i>D. repens</i> (reflux extraction)	15.51	13	9	0	11
DMSO	0	0	0	0	0
<i>F. piperis</i> (reflux extraction)	21.90	0	0	0	0
<i>F. piperis</i> (macerating extraction)	7.39	0	0	0	0
<i>H. candicans</i> (macerating extraction)	5.94	0	0	0	0
<i>H. candicans</i> (reflux extraction)	21.45	9	0	0	12
<i>H. capitellata</i> (macerating extraction)	3.02	0	0	0	0
<i>H. capitellata</i> (reflux extraction)	8.75	0	0	0	0
<i>I. simonsii</i> (macerating extraction)	5.76	15	0	0	0
<i>I. simonsii</i> (reflux extraction)	10.14	10	0	0	16
<i>L. santali</i> (macerating extraction)	9.53	0	9	0	0
<i>L. santali</i> (reflux extraction)	16.91	0	0	0	13
<i>M. pachycarpa</i> (macerating extraction)	22.17	0	0	0	0
<i>M. pachycarpa</i> (reflux extraction)	40.97	0	0	0	0
<i>M. tinctoria</i> (macerating extraction)	7.94	22	9	0	20
<i>M. tinctoria</i> (reflux extraction)	14.47	22	0	0	11
<i>N. indicum</i> (macerating extraction)	8.75	0	0	0	12
<i>N. indicum</i> (reflux extraction)	29.29	0	0	0	0
<i>P. henryi</i> (macerating extraction)	7.23	23	0	0	15
<i>P. henryi</i> (reflux extraction)	4.88	27	12	0	0
<i>R. curcuma</i> (macerating extraction)	1.75	0	0	0	0
<i>R. curcuma</i> (reflux extraction)	9.25	0	0	0	16
<i>R. uncaria</i> (macerating extraction)	7.82	13	0	0	0
<i>R. uncaria</i> (reflux extraction)	23.33	16	0	0	0
<i>S. baccatum</i> (macerating extraction)	4.62	20	0	0	13
<i>S. baccatum</i> (reflux extraction)	16.95	16	10	0	11
<i>S. discolor</i> (macerating extraction)	16.05	0	0	0	0
<i>S. discolor</i> (reflux extraction)	9.03	15	0	0	0
<i>T. ciliata</i> Roem. var. pubes (reflux extraction)	18.92	0	0	0	0
<i>T. ciliata</i> Roem. var. pubescens (Franch.) Hand.-Mazz. (macerating extraction)	3.99	12	0	0	0

SA: *Staphylococcus aureus* (ATCC25923); EC: *Escherichia coli* (ATCC25922); PA: *Pseudomonas aeruginosa* (ATCC27853); CA: *Candida albicans* (ATCCY0109).

The anti-MRSA activities of the selected 17 extracts which were active with IZDs ≥ 10 mm against MSSA were shown in Table 4. The extracts from *Psychotria*

henryi Levl and *Marsdenia tinctoria* R. Br. (maceration and reflux extraction) were the most active against both SA and MRSA, with mean IZDs = 19.7 to 22.0 mm (Table 4).

Table 4. The testing results of the 17 extracts with IZD $\geq$ 10mm against SA and MRSA strains (unit: mm).

Species	SA	MRSA92	MRSA98	MRSA111	Mean $\pm$ SEM (MRSA, n=3)
<i>P. henryi</i> (reflux extraction)	27	25	24	17	22.0 $\pm$ 2.5
<i>M. tinctoria</i> (macerating extraction)	22	20	23	21	21.3 $\pm$ 0.9
<i>M. tinctoria</i> (reflux extraction)	22	21	19	22	20.7 $\pm$ 0.9
<i>P. henryi</i> (macerating extraction)	23	21	17	21	19.7 $\pm$ 1.3
<i>S. baccatum</i> (macerating extraction)	20	19	16	18	17.7 $\pm$ 0.9
<i>S. baccatum</i> (reflux extraction)	16	16	24	14	18.0 $\pm$ 3.1
<i>I. simonsii</i> (macerating extraction)	15	17	22	14	17.7 $\pm$ 2.3
<i>R. uncariae</i> (reflux extraction)	16	18	19	14	17.0 $\pm$ 1.5
<i>C. bejolghota</i> (reflux extraction)	16	19	15	15	16.3 $\pm$ 1.3
<i>I. simonsii</i> (reflux extraction)	10	16	16	15	15.7 $\pm$ 0.3
<i>S. discolor</i> (reflux extraction)	15	15	17	12	14.7 $\pm$ 1.5
<i>C. tigilium</i> (reflux extraction)	13	16	17	0	11.0 $\pm$ 5.5
<i>D. repens</i> (macerating extraction)	11	10	10	13	11.0 $\pm$ 1.0
<i>D. repens</i> (reflux extraction)	13	14	15	0	9.7 $\pm$ 4.8
<i>C. tigilium</i> (macerating extraction)	12	0	0	12	4.0 $\pm$ 4.0
<i>R. Uncariae</i> (macerating extraction)	13	0	0	10	3.3 $\pm$ 3.3
<i>T. ciliata</i> (macerating extraction)	12	0	0	0	0.0 $\pm$ 0.0
DMSO	0	0	0	0	0.0 $\pm$ 0.0

SA: *Staphylococcus aureus* (ATCC25923); MRSA: methicillin-resistant *Staphylococcus aureus*.

The anti-SA and MRSA active extracts were further determined for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by serial dilution with a standard broth microdilution method. 12 extracts showed effects on both SA and MRSA strains with MICs/MBCs ranges between among 32 to $\geq$ 1024 / 64 to $\geq$ 1024 mg/L, respectively. *P. henryi*, *M. tinctoria* and *Sapium baccatum* Roxb also showed inhibition against EC, PA and CA strains, and displayed a broad spectrum of antimicrobial activities.

DISCUSSION

The screening results revealed that most of the plant extracts were much more active against Gram positive pathogen (SA) than those of Gram negative pathogens (EC and PA), which is a common phenomenon of plant antimicrobial property (Tegos et al., 2002). None of the plants showed strong inhibition against PA (Table 3). The 4 plant extracts from *D. repens* (reflux extraction), *M. tinctoria* (macerating extraction), *S. baccatum* (reflux extraction) and *P. henryi* (reflux extraction) showed IZDs of 9 to 12 mm against standard EC. There were 15 plant extracts active against standard CA and the IZDs were in the ranges of 10 to 20 mm. Some plants showed a wide spectrum of antimicrobial activity and were in agreement with their folk uses (Table 3). However, several extracts showed no antimicrobial activity at 50 mg/ml which were

not consistent with their folk uses for infectious diseases. It might be due to the antimicrobial potency which was much lower in this study and the different criterion for the antimicrobial judgment. We also noted the inconsistent values between IZD and MIC of an extract; this might be due to the different diffusion capacity of the active constituents in the culture media of agar and broth.

It was found that different extraction methods produced different yields of active extracts. Meanwhile, the higher temperature of extraction may cause the active ingredient change, hence the antimicrobial activity decreases (Tables 3 to 5).

In this study, the 80% ethanol extracts of 20 plant materials were screened for their antimicrobial activities for the first time. We found that *P. henryi* was active against not only Gram-positive bacteria but also Gram-negative bacteria. The chemical composition and pharmacological effects of *P. henryi* has not been reported yet, but there were reports (Muhammad et al., 2003; Kerber et al., 2001) about the same genus, that is, *P. sychotria brachyceras* and *P. sychotria klugii* which contained indole alkaloids. So, it is worthy to further investigate the anti-MRSA components. Our study suggested that *M. tinctoria* showed promising activity against MSSA and MRSA. The plant contained steroidal components such as tinctoralactone and marsdenone (Chowdhury et al., 1993) and showed antifertility activity (Chowdhury et al., 1994). But its anti-MRSA effect has not been noted previously. Further research for the corresponding active

Table 5. MICs and MBCs of the 12 extracts with IZD $\geq$ 10mm against SA, MRSA and PA strains (unit: mg /L).

Strain	Parameter	PA	ATCC 25923	MRSA 092	MRSA 098	MRSA 111
<i>P. henryi</i> (reflux extraction)	MIC	1024	32	64	128	32
	MBC	n.a.	64	128	512	64
<i>S. baccatum</i> (macerating extraction)	MIC	n.a.	32	512	128	32
	MBC	n.a.	128	1024	512	128
<i>R. Uncariae</i> (reflux extraction)	MIC	n.a.	64	64	512	1024
	MBC	n.a.	256	256	1024	2048
<i>S. baccatum</i> (reflux extraction)	MIC	2048	64	512	256	64
	MBC	n.a.	128	1024	512	256
<i>P. henryi</i> (macerating extraction)	MIC	2048	128	128	512	512
	MBC	n.a.	1024	1024	2048	2048
<i>M. tinctoria</i> (macerating extraction)	MIC	n.a.	128	128	1024	64
	MBC	1024	512	512	2048	512
<i>M. tinctoria</i> (reflux extraction)	MIC	n.a.	256	256	1024	64
	MBC	1024	1024	1024	2048	512
<i>I. simonsii</i> (macerating extraction)	MIC	n.a.	512	256	256	512
	MBC	1024	1024	512	512	1024
<i>I. simonsii</i> (reflux extraction)	MIC	n.a.	1024	256	512	1024
	MBC	n.a.	2048	512	1024	2048
<i>D. repens</i> L. (macerating extraction)	MIC	n.a.	1024	1024	1024	512
	MBC	n.a.	2048	2048	2048	2048
<i>C. bejolghota</i> (reflux extraction)	MIC	n.a.	1024	1024	512	1024
	MBC	n.a.	2048	2048	2048	2048
<i>S. discolor</i> (reflux extraction)	MIC	n.a.	1024	512	256	512
	MBC	n.a.	2048	2048	2048	2048
Vancomycin	MIC	0.244	0.244	0.488	0.244	0.488
	MBC	0.488	0.488	0.976	0.976	0.976

SA: *Staphylococcus aureus* (ATCC25923); MRSA: methicillin-resistant *Staphylococcus aureus*; PA: *Pseudomonas aeruginosa* (ATCC27853); n.a.: not active at the concentration up to 2048 mg/L.

components from these plants and their systematic anti-MRSA properties are been carried out.

81073126, 81173504).

ACKNOWLEDGEMENT

This research was financially supported by the grant from the National Natural Science Foundation of China (NSFC

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