Full Title: Antimicrobial and antioxidant activities of traditional Thai herbal remedies for aphthous ulcers
Short Title: Antimicrobial activity of Thai herbal remedies for aphthous ulcer **Manuscript Type:** Original paper **Order of Authors:** Chantana Mekseepralard[a,*], Narisa Kamkaen[b], and Jenny M Wilkinson[c a] Faculty of Medicine, Srinakharinwirot University, Klongtoei, Wattana, Bangkok 10110, Thailand [b] Faculty of Pharmacy, Srinakharinwirot University, Ongkharak, Nakhon-Nayok, 26120, Thailand [c] School of Biomedical Sciences, Charles Sturt University, Wagga Wagga NSW 2678 Australia ***Corresponding Author:** Chantana Mekseepralard, Ph.D. **Corresponding Author's Institution:** Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Klongtoei, Wattana, Bangkok 10110, Thailand E-mail address: chantana@swu.ac.th Tel.: +662-6641000 ext. 4917; Fax: +662 6641000 ext. 4902 **Research grant:** The National Research Council of Thailand (NRCT)

Abstract

Four medicinal plants (*Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis* and *Glycyrrhiza uralensis*) as well as one traditional Thai treatment for aphthous ulcers based on these four plants were tested for antimicrobial activity. MIC values for a range of bacteria and *Candida albicans* were determined, with both type strains and clinical isolates being used. Antioxidant activity was determined using the ABTS radical scavenging assay. Among four the plants, *Q. infectoria* showed antimicrobial activity against *Staphylococcus aureus* with an MIC of 0.41 mg/mL while *C. chinensis* showed antifungal activity against

C. albicans with an MIC of 6.25 mg/mL. Activity was also shown against a range of other organisms including *Salmonella typhi*, *Serratia marcescens*, *Vibrio cholerae*, *Vibrio parhaemolyticus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis.* The antimicrobial activity of the traditional aphthous ulcer preparation (a powder) was comparable to that for the individual plants extracts however incorporation of the powder into a gel formulation resulted in a loss of almost all activity. All extracts, with the exception of *K. galanga*, also showed good antioxidant activity. This study supports the traditional use of these plants and suggests that they may also be useful in the treatment of other infections.

Keywords: *Quercus infectoria*; *Kaempferia galanga*; *Coptis chinensis*; *Glycyrrhiza uralensis*; Antimicrobial activity; Antioxidant activity

Introduction

Recurrent aphthous ulcer (RAU) is the most common chronic, painful oral condition in otherwise healthy patients and causes considerable discomfort. While the exact cause of these ulcers is not well established there are many factors that provoke ulcer formation including stress, fatigue, illness, injury from accidental biting, and hormonal changes such as during the menstrual cycle (Vucicevic Boras and Savage, 2007; Wray *et al*., 1978). While normally a self-limiting condition that resolves within 1-2 weeks, symptomatic treatment with oral antibiotics, local anesthetics and anti-inflammatory medication may be used (Mcbride, 2000). In the case of severe ulcers treatment with corticosteroids is suggested (Scully, 2006; Scully and Shotts, 2000). The use of plant extracts to treat a wide range of conditions is well known and the treatment of oral and dental conditions is no exception. For example *Alchemilla vulgaris,* chamomile (*Matricaria chamomilla*) and *Aloe barbdensis* have all been used in the treatment of aphthous ulcers (Sahba and Mohammadalipour, 2005; Shrivastava and John, 2006; Vucicevic Boras and Savage, 2007; Wynn, 2005).

In Thailand, there are a number of traditional plant-based remedies that have been used for the treatment of aphthous ulcers. The treatment in this study comprises extracts from the following plants *Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis* and *Glycyrrhiza uralensis*. The galls that form on the branches of the tree *Q. infectoria* following gall-wasp attack are thought to have a number of pharmacological actions including local anesthetic, antimicrobial and antipyretic affects. The galls consist of a large amount of tannins which have shown potential antiviral, antibacterial and antiparasitic effects. In Asian countries, the galls have been used for treating inflammatory diseases and gargling a hot water extract of the galls is thought to be effective for tonsillitis while ointments containing the powdered galls are used for skin inflammation (Basri and Fan, 2005; Kaur *et al*., 2004).

K. galanga, *C. chinensis* and *G. uralensis* are also used in traditional Chinese medicine. Decoctions and other products comprising powdered rhizomes of *K. galanga* are used for digestive problems, the common cold, headaches, toothaches, and pectoral and abdominal pains (Kanjanapothi *et al*., 2004). Animal studies by Sulaimann *et al*. (2008) also found that the aqueous extract has antinociceptive and anti-inflammatory actions providing supporting evidence for the traditional use of this plant. The rhizomes of *C. chinensis* are used in traditional Chinese medicine as an antipyretic, and for the treatment of diarrhea, dysentery, jaundice, seasonal feverish diseases, sore eyes and sore throat. The major active ingredient, berberine, is an isoquinoline derivative alkaloid and has many pharmacological effects (Liu *et al*., 2006).

In traditional Chinese medicine the roots and rhizomes of licorice (*Glycyrrhiza uralensis Fisch. ex DC.; "Cha Em Jean" in Thai; "Chinese Licorice" in general)* have many reported biological activities including anti-ulcer, antipyretic, antimicrobial, antiviral, anti-inflammatory, antidepressant, antioxidant activities, expectorant, diuretic, laxative, sedative, and menopausal complaints (Hu *et al*., 2009). Chemical studies of *G. uralensis* have identified the main constituent as glycyrrhizin, a triterpenoid saponin glycoside of glycyrrhizic acid, along with numerous phenolic compounds. The phenolic compounds are predominantly isoprenoid-substituted phenols (isoflavans with oxygen substitutions at the C-5 position) and which have been demonstrated to have antibacterial activity (He *et al*., 2006; Hatano *et al*., 2000). Glycyrrhizin, also has biological activity primarily antiviral, anti-inflammatory, and antioxidant actions (Dhingra *et al*., 2004). Burgess *et al*. (2008) have also reported on the potential of CankerMelts® GX patches (Orahealth Corporation) which contain 30 mg of a glycyrrhiza extract to reduce the pain associated with aphthous ulcers. As these four plant products have been used traditionally in Thailand for the treatment of aphthous ulcers, and infection has been associated with the

inset of symptoms in some individuals, this study was therefore designed to investigate the antimicrobial activities of a traditional Thai aphthous powder and its four main components.

Materials and methods Acquisition of chemicals, plant materials and microorganisms

All chemicals were purchased from DIFCO (Detroit, MI) and Sigma Chemicals (St. Louis, MO, USA). The dried and pulverized galls of *Q. infectoria* (Olivier), roots of *G. uralensis* (Fisch.), rhizomes of *K. galanga* (Linn.), and roots of *C. chinensis* (Franch) were kindly provided by Khaolaor Bhaesaj Ltd., Part, Thailand. Voucher specimens were deposited in the Pharmacognosy Department Herbarium, Faculty of Pharmacy, Srinakharinwirot University, Thailand. The aphthous powder was provided by the manufacturer, Khaolaor Bhaesaj Ltd., Thailand. The aphthous gel was developed by using carbopol 934P as the gelling agent and consisted of 10% w/w of aphthous powder. For the antimicrobial assays the dry, powdered plant material and aphthous powder were dissolved in dimethylsulfoxide (DMSO) at 100 mg/mL immediately prior to use. The aphthous gel and gel base were also prepared in DMSO to a final concentration of 1 g/mL.

Type strains of *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes* (DMST 17020) and *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Salmonella typhi* (DMST 5784), *Serratia marcescens* (ATCC 8100), *Vibrio cholerae* (non 01, non 0139, DMST 2873), *Vibrio parahaemolyticus* (DMST 5665), *Enterococcus faecalis* (ATCC 29212) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. In addition five clinical isolates of *S. aureus* and of *C. albicans* were obtained from the Division of Clinical Microbiology, Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Nakhon-nayok, Thailand. A further four clinical isolates of *S. aureus* were obtained from the Microbiological Section, Queen Sirikit National Institute of Child Health, Bangkok, Thailand and 5 clinical isolates of *C. albicans* and of *S. pyogenes* (group A beta-hemolytic streptococci) were provided by the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand.

Screening for antifungal activity

Antifungal activity was determined using the agar well diffusion method (Zaidi and Crow, 2005) with some modifications. Ten clinical isolates of *C. albicans* and a standard reference strain (ATCC 10231) were first grown on Sabouraud dextrose agar (Himedia, India) plate at 30°C for 24-48 h. One isolated colony was inoculated into 2 mL of Sabouraud dextrose broth and the culture was then incubated with shaking at 30°C until adequate growth was noticed. The culture inocula were adjusted with sterile saline to match turbidity equivalent to a 0.5 McFarland standard solution. These inocula were spread on to Sabouraud dextrose agar plates in three directions to ensure that the complete distribution of the inocula. Wells, 6 mm in diameter, were formed on to the media using a sterile metallic borer and 100 μL of each

of the test agents were added into the wells and the plates allowed to rest at room temperature for 30-60 min. Plant extracts were used at concentrations ranging from 100 – 3.125 mg/mL and the aphthous gel and gel base were tested at concentrations of 500 and 1,000 mg/mL. Zones of inhibition were measured in mm following incubation of the plates at 30°C for 24 -48 h. DMSO (100%) and gel base were used as negative controls. All tests were carried out in duplicate. The minimum inhibitory concentration (MIC) was deemed to be the lowest concentration of the test agent which inhibited the growth of microorganisms.

Screening for antibacterial activities

Antibacterial assays were carried out using the agar dilution method (Forbes *et al*., 2002) with the testing conditions as per those recommended by the Clinical and Laboratory Standard Institute (2005). Fresh bacterial cultures were prepared on blood agar plates and grown at 35°C for 18-24 h. For assays one to two isolated *S. aureus* colonies were transferred into a tube containing 2 mL of nutrient broth (Himedia, India) while for *S. pyogenes* five or six colonies were added to 2 mL of brain heart infusion broth (BHI; Difco USA). The tubes were incubated with shaking at 35°C for 4-6 h and then adjusted with sterile saline to match the 0.5 McFarland standard solution. The culture was then diluted 1:100 in sterile saline (or in BHI broth for *S. pyogenes*) to reach the bacterial density of $1.5x10^{6}$ CFU/mL. All incubations for *S. pyogenes* were in a 5% CO_2 atmosphere.

Mueller Hinton agar (MHA) plates were prepared by adding 6 mL of cooled, molten agar (or molten MHA included 5% sheep red blood cells for *S. pyogenes*) to test tubes containing 1 mL of plant extract (6.5-0.026 mg/mL). The mixture of the media and the plant extracts was poured into sterile petri dishes and the media allowed to solidify before use. Two control tubes containing 1 mL of either sterile water or DMSO at the same concentration as in the test media were prepared in parallel to serve as controls. The plates were dried at room temperature and 10 µL of bacterial inoculum ($1.5x10^4$ CFU) was inoculated onto the agar plates and incubated at 35°C for 18-24 h. The plates were then examined for bacterial growth and the MIC was the lowest concentration of the plant extract in agar that was devoid of visible growth. Gentamicin was included as a positive control. All tests were carried out in duplicate.

Antioxidant assay

Antioxidant potential was determined using the ABTS (2,2'-azinobis(3-ethylbenzthiazoline6-sulphonic acid)) radical scavenging assay. Plant material was immersed in 95% ethanol at the ratio of 1:8 (w/v) and shaken (250 rpm) for 72 h at room temperature (37°C). The containers were wrapped with aluminum foil to protect them from light and the material was subjected to vacuum filtration. The resultant liquid was used in the assay.

The pre-formed radical monocation of 2, 2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was generated according to the modified method of Re *et al*. (1999) by oxidation of ABTS solution (7 mM) with 2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 12 h in the dark at room temperature (37°C). The ABTS solution was diluted with ethanol to the absorbance of 0.7 ± 0.2 at 750 nm. A series of extract solutions were prepared by dissolving the test sample in ethanol and 20 µL of each ethanolic solution was then added to 180 µL of $ABTS^{\cdot+}$ radical cation solution. The absorbance was monitored spectrophotometrically at 734 nm for 60 min by using a microtitre plate reader. All measurements were performed in triplicate. An IC_{50} was then calculated from the resulting data.

Results

The plant materials were evaluated for antimicrobial activity against type and clinical isolates of a range of bacteria and the yeast *C. albicans*. All four plant extracts were active against *C. albicans* with MICs ranging from 6.25 to 100 mg/mL (Table 1). The activity of the extracts was variable with, overall, *Q. infectoria* demonstrating the weakest activity (MICs 50-100 mg/mL). The activity of the commercial aphthous powder was within the same MIC range (25-100 mg/mL) as the plant extracts; however the formulation of the powder into a gel significantly reduced the powder's activity against this yeast (MIC > 1,000 mg/mL).

Both *S. aureus* and *S. pyogenes* were susceptible to the plant extracts with little difference in susceptibility between the type strain and clinical isolates (Tables 2 and 3 respectively).

The activities of *G. uralensis*, *Q. infectoria* and *K. galanga* were comparable for both bacteria (MIC range 0.41-0.813 mg/mL for *S. aureus* and 1.625-6.5 mg/mL for *S. pyogenes*). The MIC of these three extracts against *S. aureus* were lower than that of the *C. chinensis* extract and the aphthous powder (MIC 3.25 ->6.5mg/mL). In contrast *C. chinesis* was shown to have better activity against *S. pyogenes* (MIC 0.41-0.813 mg/mL) while the aphthous powder was comparable to that of the other three extracts.

There were few differences in activity against the remaining bacteria with most extracts and the aphthous powder showing MICs of 0.813-6.5 mg/mL or greater (Table 4). *Q. infectoria* had the strongest activity against *S. marcescens, S. typhi*, *V. cholerae*, *V. parhaemolyticus*, *P. aeruginosa* and *E. faecalis*. *G. uralensis* also showed good activity against *E. faecalis* (MIC 0.813 mg/mL) and the aphthous powder good activity against *P. aeruginosa* (MIC 0.813) and *S. marcescens* (0.813 mg/mL).

The strongest ABTS radical scavenging activity was shown by *Q. infectoria* (IC_{50} 10.03 μg/mL), followed by *C. chinensis* (159.02 μg/mL) and *G. uralensis* (169.18 μg/mL). The IC_{50} of *K. galanga* was unable to be determined as, in this assay, the extract did not show any free radical scavenging activity.

Discussion

Aphthous powder, a Thai herbal remedy, has traditionally been claimed as a treatment for aphthous ulcers; however, the antimicrobial efficacy of this product has not been investigated. Extracts of *Q. infectoria*, *K. galanga*, *C. chinensis*, *G. uralensis*, the four main components of the powder, and the powder were tested for their antimicrobial potential against a range of organisms. The results

demonstrated that Gram positive bacteria were more susceptible than *C. albicans*. *Q. infectoria*, *K. galanga*, and *G. uralensis* had a stronger inhibitory effect against ten strains of *S. aureus*, with MIC values of 0.41-0.813 mg/mL. In addition antioxidant activity was demonstrated for three of the four plant extracts.

Previous studies have also reported the antimicrobial properties of the plants used in this study showing MIC values, where reported, comparable to those found in this study. *Q. infectoria* extracts have been shown to be effective against *E. coli*, *Bacillus subtilis*, *K. pneumoniae*, *Proteus vulgaris* and *P. aeruginosa* (Basri and Fan, 2005; Singh *et al*., 2005), *Entamoeba histolytica* (Sawangjaroen *et al*., 2004) and it also inhibited hepatitis C virus protease (Hussein *et al*., 2000). Both ethanolic and aqueous extracts of *Q. infectoria* were the most effective against *E. coli* O157:H7 with MIC and MBC values of 0.09 and 0.78 mg/mL, respectively (Voravuthikunchai *et al*., 2004). As they are the main compound in galls of *Q. infectoria*, tannins may play an important role in the antimicrobial mechanisms of this extract. It has been reported that tannins with astringent properties may induce complex formation with enzymes or substrates and its toxicity may result from tannin action on the membranes of microorganisms (Akiyama *et al*., 2001).

Fukai *et al*. (2002a) in their study of flavonoids isolated from three licorice *Glycyrrhiza* species *(G. glabra*, *G. inflata* and *G. uralensis*) found that licoisoflavone B and ethanol extracts of *G. uralensis* root exhibited activity against both methicillin-susceptible *S.*

aureus (MIC of 1.56-12.5 µg/mL) and methicillin-resistant *S. aureus* (MIC of 1.56-25 µg/mL). These compounds were also active against *Micrococcus luteus* and *B. subtilis* (MIC of 3.13-25 µg/mL), but not Gram negative bacteria such as *E. coli*, *K. pneumoniae* and *P. aeruginosa*. One of the ethanol extracts, licoricidin, showed a remarkable activity in decreasing the resistance of MRSA to amoxicillin by affecting the enzymatic function, but not the formation, of penicillin-binding protein 2' (Hatano *et al*. 2000). However, licoisoflavone B and licoricidin also exhibited inhibitory activity against the growth of *Helicobacter pylori*, a causative agent of peptic ulcer (Fukai *et al*. 2002b).

The antimicrobial activity of members of the Zingiberaceae family is well known however there are very few studies that report specifically on the activity of *K. galanga*, and most report on activity of the essential oil rather than extracts (Chen *et al*., 2008; Parvez *et al*., 2005; bin Jantan *et al*., 2003; Arambewela *et al.,* 1999). In this study *K. galanga* extracts were active against both bacteria and *C. albicans* suggesting that the activity observed in other Zingiberaceae is also seen in *K. galanga* and may be due to a common chemical constituent. Similarly there is little published literature on the antimicrobial effect of *C. chinensis* extracts however it is thought that any antimicrobial activity would be due to the presence of berberine. Berberine chloride, an isoquinoline alkaloid derivative extracted from rhizomes of *C. chinensis,* has an inhibitory effect on the sortase protein of Gram-positive bacteria (Kim *et al*., 2004). This protein is important for the pathway involved in the secretion and anchoring of cell wall proteins. The results of this study showed that although the extract of *C. chinensis* has weak antibacterial activity, it produced the greatest effect on *C. albicans* with the MIC values varying from 6.25-25 mg/mL. In contrast, Yu and colleagues (2005) have reported that berberine shows activity against methicillinresistant *S. aureus* with MIC values ranging from 32-128 µg/mL.

In this study the four aforementioned plant products were also mixed into a commercial aphthous powder that is based on a traditional Thai recipe. The powder showed good antibacterial and anticandidial activity with MICs slightly higher but comparable to those for the individual components. The powder comprises a range of herbal extracts which includes 23% *Q. infectoria*, 17% *K. galanga*, 4% each of *G. uralensis* and *C. chinensis* (Khaolaor Bhaesaj Ltd., Thailand). However when mixed into a gel the antibacterial activity was significantly reduced perhaps due to an interaction between the gel and the powder, trapping of active components within the gel matrix or the low concentration of powder within the gel (gel contains 10% w/v of the powder). From these overall results, we can conclude that aphthous powder, a traditional Thai herbal remedy, containing the four main components of plant materials used for aphthous ulcer possesses antimicrobial effects against a range of pathogenic bacteria and *C. albicans.* This study has also demonstrated the antioxidant activity of three of the four individual plant extracts (no activity was found with *K. galanga*) which may contribute to the anti-inflammatory activity of these extracts. Together the demonstrated antimicrobial and antioxidant activity of these extracts support the traditional use of these plant products and suggest that there is a potential for the treatments of not only aphthous ulcers but also other topical infections caused by the microorganisms examined in this study.

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Table 1. Minimum inhibitory concentration (MIC) of 4 plant extracts, aphthous powder and aphthous gel against 10 *C. albicans* clinical isolates and a standard strain of *C. albicans* ATCC 10231. No inhibition of fungal growth was observed in control wells.

	MIC (mg/mL)										
		ATCC	**Clinical isolates**								
		1023	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
G. uralensis	25	25	25	25	25	25	12.5	25	25	25	25	25
Q. infectoria	50	100	50	50	50	50	100	100	100	100	100	100
K. galanga	25	50	25	50	25	50	12.5	50	50	50	50	50
C. chinensis	12.5	6.25	6.25	12.5	12.5	12.5	25	25	25	25	25	25
Aphthous powder	25	100	25	25	50	50	100	50	50	100	50	50
Aphthous gel	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000

Table 2. Minimum inhibitory concentration (MIC) of plant extracts and aphthous powder against nine clinical isolates and a type strain of *S. aureus.* No inhibition of bacterial growth was observed in control plates.

	MIC (mg/mL)									
	Herbal	ATCC	clinical isolates							
extracts		25923	1	2	3	4	5	6	7	8
G. uralensis		*0.813*	*0.813*	*0.813*	*0.813*	*0.813*	*0.813*	*0.41*	*0.41*	*0.813*	*0.813*
Q. infectoria		*0.41*	*0.813*	*0.813*	*0.813*			*0.813*	*0.813*	*0.41*	*0.41*	*0.41*	*0.813*
K. galanga		*0.813*	*0.813*	*0.813*	*0.813*	6.5	6.5	6.5	6.5	*0.813*	*0.813*	*0.41*	*0.41*	*0.813*	*0.813*	6.5	6.5
C. chinensis		6.5	3.25														
Gentamicin		0.25	ND	ND	ND	ND	ND	ND	ND	ND	ND	(μg/mL)					
Aphthous powder		3.25	6.5	3.25	3.25	6.5	6.5	3.25	3.25	3.25	3.25						

Table 3. The minimum inhibitory concentration (MIC) of plant extracts, and aphthous powder against *S. pyogenes* (Group A beta-hemolytic *Streptococci*), reference strain and five clinical isolates. No inhibition of bacterial growth was observed in control plates.

	MIC (mg/mL)					
	DMST 17020	**clinical isolates**				
	1	**2**	**3**	**4**	**5**	
G. uralensis	*6.5*	*3.25*	*1.625*	*3.25*	*3.25*	*3.25*
Q. infectoria	*6.5*	*3.25*	*3.25*	*3.25*	*3.25*	*3.25*
K. galanga	*6.5*	*3.25*	*1.625*	*3.25*	*3.25*	*3.25*
C. chinensis	*0.813*	*0.813*	*0.41*	*0.813*	*0.41*	*0.4*1
Aphthous powder	> 6.5	6.5 >	6.5 >	6.5	6.5	6.5

Table 4. Minimum inhibitory concentration (MIC) of plant extracts and aphthous powder against a range of organisms. No inhibition of bacterial growth was observed in control plates.

	G. uralensis	*Q. infectoria*	*K. galanga*	*C. chinensis*	Aphthous powder	Gentamicin (μg/mL)	MIC (mg/mL)
G. uralensis	*Q. infectoria*	*K. galanga*	*C. chinensis*	Aphthous powder	Gentamicin (μg/mL)		
Escherchia coli	>6.5	>6.5	>6.5	6.5	>6.5	1.0	
Klebsiella pneumoniae	>6.5	>6.5	>6.5	>6.5	6.5	8.0	
Salmonella typhi	>6.5	0.41	>6.5	6.5	6.5	0.5	
Seratia marcescens	6.5	0.05	6.5	6.5	0.813	4.0	
Vibrio cholerae	>6.5	0.813	>6.5	3.25	6.5	1.0	
Vibrio parahaemolyticus	>6.5	0.41	>6.5	6.5	1.625	2.0	
Enterococcus faecalis	0.813	0.813	1.625	6.5	>6.5	8.0	
Pseudomonas aeruginosa	>6.5	0.41	>6.5	6.5	0.813	1.0	