Antibacterial Activity of the Seeds of *Combretum quadrangulare* Kurz (Combretaceae)

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ABSTRACT

Chemicals from seeds of Combretum quadrangulare Kurz (Combretaceae) were extracted with methanol. Purified crude methanolic extracts were done by microcrystalline cellulose column chromatography and microcrystalline cellulose preparative layers 2 times by using MeOH-H_2O (1:1) as the developing solvent.

It was found that crude methanolic extracts of 2 samples (one collected in 1997, the other in 2001), by purified column chromatographic extraction and preparative layer chromatographic residue showed antibacterial activity against gram-positive cocci and non-fermentative gram-negative bacilli better than fermentative gram-negative bacilli. From spectroscopic data and chemical tests, it was found that the isolated residues from preparative layer chromatography were steroids and flavonoid glycosides.

MIC (mininum inhibitory concentration) of four sample groups for sensitive bacteria were nearly of the same level so we can use the seeds of Combretum quadrangulare Kurz directly without purification. The seeds can be used for several years if they are kept in an air-tight and dry condition.

Key words: *Combretum quadrangulare* Kurz., Antibacterial activity

INTRODUCTION

Combretum quadrangulare Kurz. is found throughout Thailand especially in open, wet places. Therapeutic uses of this plant in the country are for anthelmintics (the parts used were seeds, roots and leaves) and curing venereal disease (the parts used are roots and wood) (Pongbunrods, 1979).

Somanapun et al., (1980) studied the chemical constituents of this plant. They found that alcoholic and other extracts from the roots and seeds could kill earthworms. They also found that crude extracts from seeds showed antibacterial activity. The flavonoid compound found in this plant is combretol. They further found new compounds from roots and seeds which were 3 compounds of pentacyclic triterpene carboxylic acid, viz., 3β, 6β, 18β-trilrydroxy-urs-12-en-30-ic acid, 3,6-diketo-olean-12-en-28-oic acid and olean-12-en-28-oic acid. They also found β-sitosterol and β-sitosteryl, 2 compounds of long-chain alcohol and amino compound.

Castledon et al.(1985) found three flavonoids from the flowers of this plant which were:

1) 5-hydroxy-3,7-dimethoxy-2-(3',4',5'-trimethoxyphenyl)-4H-1-benzopyran-4-one (combretol)

2) 5-hydroxy-2-(3'-hydroxy-4'methoxyphenyl)-3,7-dimethoxy-4H-1-benzopyran-4-one (ayanin) and

3) polymorphic forms of 5-hydroxy-2-(4'-hydroxy-3,5'-dimethoxyphenyl)-3,7-dimethoxy-4H-1-benzopyran-4-one

Yuvat et al., (1988) studied the anthelmintic activity of seeds of *C. quadrangulare* Kurz for roundworms in young buffalo. They found that after young buffalo ate the seeds once, the number of eggs of *Neoascaris vitulorum* in feces was reduced and completely disappeared within 1-3 weeks. They also studied the toxicity of the seeds of this plant and found that seed extracts were not toxic to albino rats and mice. The doses that they studied did not kill the rats and no side effects was found within 48 hours after giving the extract.

Ganzera et al., (1998) found 2 flavonoids, viz., kumatakenin and isokaemferide and also found 3 types of cycloartane triterpenes from the leaves of this plant which were 1α, 3β-dihydroxy-cycloart-24-ene-30-carboxylic acid, 1α, 3β-dihydroxycycloart-24-ene-30-carboxylic acid methyl ester and 1α, 3β-25-trihydroxy-cycloart-21-al-23-ene-30-carboxylic acid methyl ester.

Adnyana et al., (2000) found that MeOH, MeOH-H_2O (1:1) and water extracts of the seeds of *C. quadrangulare* Kurz. Included triterpene glycosides which showed good hepatoprotective activity.

Banskota et al., (2000) found 15 new cycloartane-type triterpenes from methanolic extracts of the leaves of this plant which showed different cytotoxicities.

Adnayana et al., (2001 a) found new gallic acid from methanolic extracts of the seeds of this plant which also showed strong hepatoprotective activity.

Adnayana et al., (2001 b) found 3 new triterpenes from methanolic extracts of the seeds of *C. quadrangulare* Kurz, being of the lupane type which included 2α, 6β-dihydroxy betulinic acid, 6β-hydroxyhovenic acid, and oleanone types which was 6β-hydroxy-arjunic acid. They also found that these compounds showed hepatoprotective activity.

From our preliminary work, we found that crude methanolic extracts from the seeds of *C. quadrangulare* Kurz showed MIC (mininum inhibitory concentration) against *Staphylococcus aureus* ATCC 25923 at 312 μg/ml and also showed antibacterial activity against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebciella pneumoniae.* We also found that crude methanolic extracts could dissolve in water. This investigation reports on the antibacterial activity of the seeds of this plant done with water-soluble compounds which is the new trend of working in phytochemistry.

MATERIALS AND METHODS

Plant material and preparation of extracts

Combretum quadrangulare Kurz specimens were collected from Chom Tong, Chiang Mai Province in the forest in 1991 and in 2001. Mature seeds were extracted (Nantachit, voucher No.2) and kept in CMU's Pharmacy Herbarium, The seeds were dried at 40°C and

powdered. Fifty grams of powder were macerated with 250 ml. of methanol. Each replicate was macerated for 1 day, filtered and repeated 2 times. The filtrate was evaporated in a vacuum. The residue (crude extract) was brownish black. The percent yield of material collected in 1997 was 36.52 and the other one was 53.22.

Determination of antibacterial activity of crude methanolic extracts by the agar well diffusion method

Two crude methanolic extracts were screened for antibacterial activity against *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 by using 10% W/V concentration of the crude methanolic extracts (Lenette, 1980; Washingon 1981).

Determination of MIC (minimum inhibitory concentration) of crude methanolic extracts against 19 pathogenic bacteria by the agar dilution method

MIC of two crude methanolic extracts against 19 pathogenic bacteria were determined by the agar dilution method (Lenette, 1980; Washington, 1981).

Determination of MIC of purified combined column chromatographic fractions of the seeds of *C. quadrangulare* Kurz against 15 pathogenic bacteria by the agar dilution method

Crude methanolic extracts were further purified by column chromatography. Microcrystalline cellulose was used as an adsorbent and the column eluted with MeOH : H_2O (1:1). A total 6 fractions, 10 ml each was collected. Each fraction was found to produce the same spot in thin layer chromatogram. Each fraction was vacuumed by vacuum pump in order to remove methanol in a cool condition because the sample fractions were heat-labile. The remaining water residue was removed by freeze-drying and the residues combined. The purified fractions were tested for antibacterial activity against 15 pathogenic bacteria by the agar dilution method (Lenette, 1980; Washington, 1981).

Determination of MIC of the residue from preparative thin layer chromatography against 10 pathogenic bacteria by the agar dilution method

Column chromatographic fractions were further purified with preparative thin layer chromatography (PTLC) twice. Microcrystalline cellulose was used as an adsorbent with 1 mm. thickness of the adsorbent developed with MeOH : H_2O (1:1). The residue from the first PTLC was separated and puridied further with the second PTLC developed in the same solvent, MeOH : H_2O (1:1). The major spot of the sample in PTLC was tested for antibacterial activity against 10 pathogenic bacteria by the agar dilution method (Lenett, 1980; Washington, 1981).

RESULTS

Antibacterial activity

Purified samples from column chromatography and from PTLC were too few and could not be tested against many pathogenic bacteria, so we reduced the number of pathogenic bacteria from 19 to 15 types for purified samples from column chromatography and also reduced from 19 to 10 types for samples from PTLC.

Crude methanolic extracts and purified samples from column chromatography and from PTLC showed antibacterial activity against gram-positive cocci (i.e., methicillin-sensitive *S. aureus* = MSSA, MIC = 1,212 μg/ml.; methicillin-resistant *S. aureus* = MRSA, MIC = 606.25 μg/ml.), non-fermentative gram-negative bacilli (i.e., *P. aeruginosa* ATCC 27853, MIC = 2,425 μg/ml.; *Acinetobacter baumanii*, MIC = 2,425 μg/ml.), and fermentative gram-negative bacilli (i.e., *E. coli*, MIC = 9,700 μg/ml).

Chemical Composition

From chemical tests, the sample gave ⊕ Libermann Burchard's reaction and also gave ⊕ Shibata reaction. These results confirmed with IR and NMR spectra.

Table 1. Antibacterial activity of crude methanolic extracts of the seeds of *C. quadrangulare* Kurz.

Descriptions	**Inhibition zone (mm)**		
	S. aureus **ATCC 25923**	***E. coli*** **ATCC 25922**	***P. aeruginosa*** **ATCC 27853**
Control (MeOH)	0	0	0
St^{d} ampicillin sodium (25 μg/ml)	37 , 37 35 , 40	0 , 0 13 , 12	0 , 0 0 , 0
average	37	12	0
St^{d} ampicillin sodium (50 μg/ml)	37 , 39 45 , 44	16 , 14 18 , 17	0 , 0 0 , 0
average	41	16	0
C. quadrangulare 10% W/V (collected in 2001)	26 , 33 29 , 28	26 , 29 27, 28	29 , 29 30, 26
average	23.9	27.5	28.5
C. quadrangulare, 10% W/V (collected in 1997)	30 , 30 28, 28	25 , 26 27, 26	22 , 22 27, 27
average	29	26	24.5

Table 2. MIC of crude methanolic extracts from purified samples by column chromatography and preparative thin layer chromatography

Pathogens	I	II	III	IV
1. *S. aureus* ATCC 25923	312	625	390.6	606.25
2. *E. coli* ATCC 25922	> 10,000	> 10,000	> 12,500	> 9,700
3. *E. coli* ATCC 35218	> 10,000	> 10,000	12,500	
4. *P. aeruginosa* ATCC 27853	1,250	2,500	1,562.5	2,425
5. MSSA	625	1,250	781.5	1,212.5
6. MRSA	312	625	390.6	606.25
7. *E. coli* ⊖	> 10,000	> 10,000	> 12,500	9,700
8. *E. coli* ⊖	> 10,000	10,000		
9. *E. coli* ⊖	> 10,000	10,000		
10. *E. coli* ⊕	2,500	10<000	> 12,500	
11. *E. coli* ⊕	5,000	10,000		
12. *E. coli* ⊕	> 10,000	> 10,000		
13. *K. pneumoniae* ⊕	2,500	5,000	> 12,500	> 9,700
14. *K. pneumoniae* ⊕	2,500	10,000	> 12,500	
15. *S. marcescens*	> 10,000	> 10,000	> 12,500	
16. *A. baumanii*	625	1,250	781.25	2,425
17. *E. cloacea*	> 10,000	> 10,000	6,250	> 9,700
18. *P. aeruginosa* Ⓢ	2,500	2,500	1,562.5	
19. *P. aeruginosa* Ⓡ	2,500	2,500	1,562.5	2,425

I = Crude methanclic extract (collected in 1997)
II = Crude methanolic extract (collected in 2001)
III = Purified sample from column chromatography
IV = Preparative thin layer chromatographic residue
MSSA = Methicillin-sensitive *Staphylococcus aureus*
MRSA = Methicillin-resistant *Staphylococcus aureus*
E. coli ⊖ = *Escherichia coli* ESBL non-producing strain
E. coli ⊕ = *Escherichia coli* ESBL producing strain
K. pneumoniae ⊖ = *Klebseilla pneumoniae* ESBL non-producing strain
P. aeruginosa Ⓢ = *Pseudomonas aeruginosa* sensitive strain
P. aeruginosa Ⓡ = *Pseudomonas aeruginosa* resistant strain

Spectroscopic data

UV spectrum

α_{Max}^{MeOH} = **225,270 and 375 nm**

IR spectrum (KBr disc)

OH-stretching polymeric,	$\nu = 3{,}410\ cm^{-1}$
Conjugated aryl C(=O) stretching,	$\nu = 1{,}720\ cm^{-1}$
C=C stretching,	$\nu = 1{,}620\ cm^{-1}$
C-O-stretching-OH-deformation of 2°-Alcohol	$\nu = 1{,}180\ cm^{-1}$
C-O-stretching-OH-deformation of 1°-Alcohol	$\nu = 1{,}080\ cm^{-1}$
CH-deformation (out of plane of disubstituted comp[d])	$\nu = 780\ cm^{-1}$

NMR Spectra (DMSO as solvent)

Flavonoid signal,	δ = 6-7 ppm
Rhamnoglucosyl sugar signal,	δ = 3-4 ppm
Sterol signal,	δ = 1-2 ppm

DISCUSSION AND CONCLUSION

Both samples purified by column chromatography and from PTLC showed antibacterial activity against gram-positive cocci and non-fermentative gram-negative bacilli better than fermentative gram-negative bacilli. These four samples showed MIC against sensitive bacteria at the same level. This investigation shows that the seeds of *C. quadrangulare* Kurz can be used directly without purification and these seeds can be used for several years if they are kept in an air-tight and dry condition.

The spectroscopic data and chemical tests showed that the active constituents in the seeds of *C. quadrangulare* Kurz are steroids and flavonoid glycosides. Further work is needed to properly identify and elucidate these compounds.

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