Xanthones from roots of *Calophyllum thwaitesii* and their bioactivity

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Abstract

Extracts from several endemic Calophyllum species were tested for antifungal, antibacterial and antioxidant activities. The crude extracts of these plants were subjected to preliminary screening for antibacterial activity against human pathogenic bacteria, Enterococci faecalis, Escherichia coli, Klebsiella, Pseudomonas aeruginosa and Staphylococcus aureus and 17 methicillin resistant Staphylococcus aureus (MRSA) strains isolated from hospitals. Antifungal activity was investigated against plant pathogenic fungi, Aspergillus and Cladosporium. Disk diffusion method was used to determine the antimicrobial activities against bacteria and the fungus Aspergillus, while TLC bioautography was used to detect the antifungal activity against Cladosporium. The results of the preliminary screening suggested that only the root methanol extract of Calophyllum thwaitesii possesed both antibacterial and antifungal activities and activity guided fractionation of this extract yielded seven xanthones. Four xanthones showed antifungal properties and were identified as 1,6-dihydroxy-5-methoxyxanthone, 1-hydroxy-5,6-dimethoxyxanthone, 1-hydroxy-5-methoxyxanthone and 1-methoxy-5-hydroxyxanthone using spectroscopic methods and comparison with literature data. Inactive compounds were identified as 1-hydroxy-7-methoxyxanthone, 1,5-dihydroxy-6-methoxyxanthone and 1,7-dihydroxyxanthone. This is the first report of above xanthones except the latter, from Calophyllum thwaitesii. Further five of the above xanthones showed free radical scavenging properties, when tested with DPPH. None of the xanthones showed prominent antibacterial activities even the crude extract did. This could possibly be due to the presence of other active minor constituents in the plant extract or some synergistic effect of combination of compounds present in the plant extract. Further, isolation of methylated xanthones in the present study suggests the presence of methylating enzymes in the root of Calophyllum thwaitesii, contrary to previous reports.

Key words: Xanthones, Calophyllum thwaitesii, Antibacterial activity, Antifungal activity, Antioxidant ctivity

Introduction

Calophyllum species are found to be a rich source of secondary metabolites such as xanthones, terpenoids, coumarins and chromene acids. However, limited attention was given to their biological activity, and in our present study we have focused on the antibacterial, antifungal activity and free radical scavenging properties of the isolates obtained from these plants. Extracts prepared from five endemic *Calophyllum* species, i.e *Calophyllum bracteatum, C. cordato oblongum, C. moonii, C. thwaitesii* and *C. zeylanicum* were preliminary screened for antimicrobial properties. Antibacterial activity was investigated against both Gram positive and Gram negative organisms which include *S. aureus* (NCTC 6571), *Enterococci faecalis* (NCTC 12697), *Pseudomonas aeruginosa* (NCTC 10662), *Klebsiella* PW *and Escherichia coli* (NCTC 10418) control strains and 17 methicillin resistant *Staphylococcus aureus* (MRSA) strains. In addition, antifungal activity was detected against plant pathogenic fungi *Cladosporium* and *Aspergillus*

However, only the methanol extract of the root stem of *C. thwaitesii* showed both antibacterial and antifungal properties. Hence this active extract was subjected to activity guided fractionation which yielded seven xanthones. Further, studies on free radical scavenging property of isolated xanthones was also carried out.

Methodology

Plant material

Calophyllum bracteatum, C. cordato oblongum, C. moonii, and C. thwaitesii were identified and collected in 1994 and 1996 from the Kanneliya forest in the Southern province of Sri Lanka, while *C. zeylanicum* was collected from Madugoda, Kandy in 1997 by Mr. Shantha Ekanayake (Institute of Fundamental Studies). Plant specimens were compared with the herbarium specimens at the Royal Botanic Gardens, Peradeniya. Dried plant materials were powdered and

extracted with hexane, methylene chloride, ethyl acetate and methanol respectively. Silica gel column chromatography, PTLC and HPLC were used to fractionate and isolate pure compounds. Structure elucidation of isolates were carried out using spectroscopic data and comparison with literature.

Antibacterial screening

Screening for antibacterial activity was carried out by Disk Diffusion method (Barry *et al.*, 1970) which is employed in most clinical microbiology laboratories for routine testing of bacterial susceptibility to chemotherapeutic agents. The principle of disk diffusion method is to allow the drug to diffuse through a surface of solid medium, concentration of the drug being highest near the site of application of drug and decreasing with distance. In the present study, sterile disk papers were loaded with required concentration of crude extract (200 μg/disk) and the disks were allowed to air dry. Thereafter the disk papers were placed on previously prepared spread plates of each organism in Mueller-Hinton Agar (MHA). Thereafter the plates were kept in a refrigerator for 1 hour and then incubated for 24 hours at 37 ^{0}C. (Sultanbawa, 1981) Readings were taken after 24 hours as zone of inhibition of growth around each disk. If the sample inhibits the pathogens growth, a clear zone or ring can be seen around the disk. The zone width is also a function of the compound's initial concentration, its solubility and its diffusion rate through agar. Chloramphenicol (SIGMA, C 7795) and Gentamicin (SIGMA, G- 3632) were used as the positive control. The effect of the solvent was also determined following the same procedure mentioned above using methanol. Above activity test for each extract was duplicated.
Thereafter, Minimum Inhibitory Concentration (MIC) of the active extracts was determined by double dilution method.(Wijesinghe, 1999) As all the crude extracts were insoluble in water, 1: 15 DMSO: H_2O solvent system was used to dissolve crude extracts. A dilution series was prepared and sterilized in MHA medium (14 mL) at 50 ^{0}C was mixed with prepared dilution series (1 mL of each concentration) in screw-capped bottles and mixed thoroughly. Then these were poured into sterile petri dishes and allowed to set on a leveled surface. Compound free control plate was also prepared. Inocula were prepared by dissolving one day old bacterial colonies (*S. aureus* NCTC 6571, *E. feacalis* and MRSA strains) in sterile distilled water and adjusting the turbidity to match with 0.5 McFarland standard. One drop of prepared inocula were inoculated on the previously prepared MHA plates and incubated for 24 hours at 37 ^{0}C. Thereafter, the plates were observed for the inhibition of growth of bacteria.

Antifungal assay

TLC bioautography (Homans, 1970) was used to check the antifungal activity of extracts against *Cladosporium*. In this method about 10 ml of Czepak Dox Broth (CDB) was added to previously prepared *Cladosporium* cultures and shook well till the water turned cloudy. Then the spores were filtered into the spraying apparatus through glass wool and the concentration of the spore suspension was adjusted to 40-50 spores per field at X 400 by adding prepared CDB medium. This spore suspension was sprayed on to previously spotted TLC/PTLC plates which were air dried for about 6 hrs. The plates were incubated at RT in a moisture chamber for 2 days and thereafter observed for inhibition in the growth.
Disk diffusion method which is commonly used for bacteria and also for yeast like fungi (Sultanbawa, 1981), was modified in order to use for filamentous fungi like *Aspergillus*. In this method a liquid culture of *Aspergillus* on CDB was prepared by inoculating seven days old fungus grown on Potato Dextrose Agar. Sterile disk papers were soaked with each extract (200 μg/disk) and left to dry completely. Meanwhile CDA medium was prepared, autoclaved and cooled to about 45^0 C and then inoculated with the liquid culture of *Aspergillus* (0.5 ml of liquid culture for 25 ml of CDA medium). Then the medium was poured into sterilized petri dishes (20 ml per each) and kept 24 hrs in the refrigerator at 4^0 C. Thereafter the plates were transferred in to an incubator (30^0 C). Readings were taken after 3 days. Diameter of the inhibition zones was measured along the two diameters at right angle to each other. Duplicate samples were used for each sample and benlate (LANKEM) was used as the positive control, while sterile distilled water was used as the negative control. Soaking the sterile disk papers in methanol and EtOAc and then following the above procedure, the effect of the solvent was also determined.

Results and Discussion

According to the preliminary investigations, methanol extract of the root stem *Calophyllum thwaitesii* has shown both antibacterial and antifungal activity. Therefore, emphasis was given to the isolation of biologically active compounds from the above plant extract. Gravity column chromatography of the methanol extract of the root stem of *C. thwaitseii* yielded thirteen fractions. Of those 5 fractions were found to be active against *Cladosporium*, and 3 were active against *Aspergillus*. Further fractionation was carried out for one of the active fraction (30 g) using hexane, EtOAc and MeOH which yielded 23 sub fractions. Further fractionation of the above sub fractions was carried out using PTLC and HPLC and were re-tested for their antifungal activity. Due to their higher and close polarities, purification was found to be very difficult even with the help of HPLC. After purification, yellow crystalline products were obtained and identification of above compounds was carried out based on physical properties (spectroscopic data) and some chemical conversions. These pure isolates were tested for their antimicrobial activities in a similar manner to that of the preliminary investigation of the crude plant extracts.

Even though the crude extract of *Calophyllum thwaitesii* root stem, had shown clear activity against *S. aureus* NCTC 6571, *E. feacalis* and MRSA strains at a concentration of 200 μg/disk, none of the pure compounds have shown antibacterial activity against any organism at the same concentration. This may be attributed to a synergetic effect of the compounds present in the crude extract. In order to evaluate the possible synergistic effect, another test was carried out using a mixture of the compounds isolated from the above crude extract. The results indicated that there was some sort of synergistic effect between these compounds for some MRSA strains, however the width of the inhibition zone was comparatively small. However, the sub fractions from which the compounds were isolated showed clear inhibition zones for *S. aureus* NCTC 6571 and MRSA strains. This might be due to the presence of other minor compounds which were not detected or lost in the isolation procedure.

However, antifungal activity studies of the pure compounds revealed that four compounds were active against both *Aspergillus* and *Cladosporium* (Table 1). After the detection of antifungal activity of pure compounds against *Cladosporium*, the MIC of active compounds was also determined using TLC bioautographic method.

^{1}H NMR spectra of above yellow crystalline compounds indicated them to be xanthones, and their CIMS showed M^{+} values as *m/z* 242, 258 and 272. Of them, the most active compound (1) had M^{+} of *m/z* 272. Its' ^{13}C NMR spectrum indicated the presence of 15 carbon atoms, including a carbonyl carbon of the xanthone moiety appeared at δ_C 182.9, two aryl methoxy groups at δ_C 57.5 and 62.1 and 12 aromatic carbon atoms. In its ^{1}H NMR spectrum, the low field 1H signal at δ_H 12.98 indicated the presence of a chelated hydroxy group adjacent to the carbonyl group at C-1, and two 3H signals at δ_H3.9 and 4.0 for the two aryl OMe groups. Further studies on HMQC and HMBC experimental data indicated that two methoxy groups to be at C-5 and C-6 of the xanthone. Considering above information, the structure of the antifungal compound (1) was proposed as 1-hydroxy-5,6-dimethoxyxanthone. The next antifungal active compound (2) had M^{+} of *m/z* 258. Its' ^{13}C NMR spectrum indicated the presence of a carbonyl carbon of the xanthone moiety appeared at δ_C 180.0, and an aryl methoxy group at δ_C 62.8. In the ^{1}H NMR spectrum, the low field 1H signal at δ_H 12.8 indicated the presence of a chelated hydroxy group adjacent to carbonyl group at C-1, and the 3H signal at δ_H4.05 for an aryl OMe group. The similarity between the ^{1}H NMR spectra of compound (1) and (2), suggested that (2) to be a monomethyl derivative of (1) and was confirmed by **TLC** comparison of (1)with methylated (2) using diazomethane. Further NMR spectral studies suggested that compound (2) to be 1,6-dihydroxy-5-methoxyxanthone. Another active compound (3) had M^{+} of *m/z* 242. Its' ^{13}C NMR spectrum indicated the presence of 14 carbon atoms, including a peak due to an aryl methoxy groups at δ_C 63.1. However, no peak was observed due to a chelated hydroxy group in its ^{1}H NMR spectrum. This observation suggested the presence of a methoxy group at C-1 of the xanthone moiety. Further studies on HMQC and HMBC experimental data confirmed that the single methoxy group to be at C-1 position of the xanthone and the structure of the antifungal compound (3) was proposed as 1-methoxy- 5-hydroxyxanthone. The next antifungal active compound (4) also had M^{+} of *m/z* 242. Its' ^{13}C NMR spectrum indicated the presence of an aryl methoxy group at δ_C 60.9. In the ^{1}H NMR spectrum of compound (4), the low field 1H signal at δ_H 12.8 indicated the

presence of a chelated hydroxy group adjacent to carbonyl group at C-1, and the 3H signal at δ_H 3.95 for an aryl OMe group. The similarity between the ^{1}H NMR spectra of compound **(4)** and compound **(3)**, suggested them to be different monomethyl derivatives from a single compound. This was confirmed by TLC comparison of methylated products of **(4)** and **(3)** which has resulted to a same compound after methylation with diazomethane. Further NMR spectral studies suggested that the 4th anti fungal compound **(4)** to be 1-hydroxy-5-methoxyxanthone. ^{1}H NMR, ^{13}C NMR, HMQC, HMBC and mass spectral data of three inactive compounds suggested them to have structures 1-hydroxy-7-methoxyxanthone **(5)**, 1,7-dihydroxyxanthone**(6)** and 1,5-dihydroxy-6-methoxyxanthone(7). (Tables 2 and 3)

In this investigation, methanol extract of the root stem of *C. thwaitesii* has been shown to contain seven xanthones (Fig. 1) including 1,7- dihydroxyxanthone**(6)**, which has previously been isolated from the bark and the timber of *C. thwaitseii*. In addition, for the first time from *C. thwaitesii*, 1-hydroxy-5-methoxyxanthone**(4)**, 1-methoxy-5-hydroxyxanthone**(3)** 1,6-dihydroxy-5-methoxyxanthone**(2)**, 1-hydroxy-5,6-dimethoxyxanthone**(1)**, 1-hydroxy-7-methoxyxanthone**(5)** and 1,5-dihydroxy-6-methoxyxanthone**(7)** have been isolated. This is the first time where methylated xanthones have been reported from *C. thwaitesii* even though some of the above methylated xanthones are reported from other *Calophyllum* species (Dharmaratne 1986).

Above Xanthones were spotted on thin layer chromatography (TLC) plates, and exposed to a free radical reagent 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Koleva 2002) to visulize anti-oxidant activity. This qualitative analysis indicated that, all the above xanthones except 1-hydroxy-7-methoxyxanthone and 1-hydroxy-5,6-dimethoxyxanthone, were found to posses radical scavenging properties thus having antioxidant activity (Table 1).

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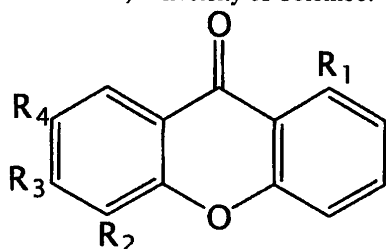

1-hydroxy-5,6-dimethoxyxanthone (1)	(R_1= OH, R_2 = R_3= OMe, R_4= H)
1,6-dihydroxy-5-methoxyxanthone (2)	(R_1= R_3= OH, R_2= OMe, R_4= H)
1-methoxy-5-hydroxyxanthone (3)	(R_1=OMe, R_2 = OH, R_3 = R_4 = H)
1-hydroxy-5-methoxyxanthone (4)	(R_1= OH, R_2= OMe, R_3 = R_4 = H)
1-hydroxy-7-methoxyxanthone (5)	(R_1= OH, R_4= OMe, R_2 = R_3 = H)
1,7-dihydroxyxanthone (6)	(R_1= R_4= OH, R_2= R_3 = H)
1,5-dihydroxy-6-methoxyxanthone (7)	(R_1= R_2= OH, R_3= OMe, R_4= H)

Figure 1. Xanthones isolated in the present study

Table 1 - Antifungal and antioxidant activities of isolated xanthones

Compound	Antifungal activity		Antioxidant activity
	Cladosporium (µg/spot)	*Aspergillus* diameter (cm)	
C. thwaitesii (root stem MeOH extract)	200	1.56	active
1,6-dihydroxy-5-methoxyxanthone	200	1.22	active
1-hydroxy-5,6-dimethoxyxanthone	50	1.07	inactive
1-hydroxy-5-methoxyxanthone	200	1.05	active
1-methoxy-5-hydroxyxanthone	200	1.00	active
1-hydroxy-7-methoxyxanthone	inactive	Inactive	inactive
1,7-dihydroxyxanthone	inactive	inactive	active
1,5-dihydroxy-6-methoxyxanthone	Inactive	Inactive	active
benalate	active	4.7	-

Table 2 - ^{13}C NMR data of isolated compounds

Carbon number	Compound						
	1	2	3	4	5	6	7
1	162.5	161.8	148.2	162.7	162.1	161.0	161.3
2	110.6	110.2	132.9	110.2	110.4	109.3	110.4
3	136.7	136.4	127.9	140.1	136.8	136.4	137.5
4	106.7	106.4	136.4		107.3	106.9	105.8
4a	156.0	155.6	157.3		156.4	156.3	
5	149.5	145.3	169.9		119.6	118.9	
6	149.2		119.3	122.0	125.9	125.1	
7	113.3	114.1	125.9	128.1	156.6	153.8	
8	121.6	123.2	115.7	129.7	104.9		121.4
8a	116.1		123.5	125.8	121.1	120.8	
9	182.9	180.0	179.1		182.3	182.2	
9a	109.4		118.0		108.9	108.1	107.0
10a	151.5		152.5		151.3	150.1	
OMe	62.1 (C-5) 57.5 (C-6)	62.8 (C-5)	63.1 (C-1)	60.9 (C-5)	56.2 (C-7)		57.2 (C-6)

Table 3 - ^{1}H NMR data of isolated compounds

Proton	Compound						
	1	2	3	4	5	6	7
1-OH	12.98 (s)	12.88 (s)		12.81 (s)	12.66 (s)	13.0 (s)	11.98 (s)
2-H	6.73 (d, J=8.25 Hz)	6.77 (dd)	7.58 (m)	7.31 (d, J=9.6 Hz)	6.75 (dd)	6.70 (dd)	6.77 (d, J=8.7 Hz)
3-H	7.52 t, J=8.25, 8.3 Hz)	7.56 (t, J=8.25 Hz)	8.24 (dd)	7.74 (t, J=8.4, 7.2 Hz)	7.53 (dd)	7.54 (m)	7.59 (t, J=8.7, 8.7 Hz)
4-H	6.81 (d, J=8.35 Hz)	6.87 (dd)	7.71 (m)	6.92 (d, J=8.4Hz)	6.86 (dd)	6.90 (dd)	6.88 (d,d)
5-H					7.33 (d, J=2.7 Hz)	7.36 (d,J=9.03Hz)	
5-OH							11.78 (s)
6-H			7.45 (d, J=8.41Hz)	7.45 (d, J=8.4Hz)	7.29 (d, J=2.8 Hz)	7.27 (m)	
7-H	7.19 (d, J=8.9 Hz)	7.20 (d, J=9.3Hz)	7.36 (t, J=9.07, , 7.64 Hz)	7.37 t, J=8.4, 7.2 Hz)			
8-H	7.38 (d, J=9.2Hz)	7.43 (d, J=9.3Hz)	7.22 (d, J=9.15 Hz)	8.28 (d, J=7.2 Hz)		7.57 (m)	7.32 (d, J=8.7 Hz)
CH_3	3.94 (s) (6-OMe) 4.02 (s) (5-OMe)	4.05 (s) (5-OMe)	3.94 (s) (1-OMe)	3.95 (s) (5-OMe)	3.89 (s) (7-OMe)		3.94 (s) (6-OMe)