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Antibacterial Activity of a Biphenyl and Xanthones from *Kielmeyera coriacea*

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Abstract

The *in vitro* antibacterial activities of the biphenyl aucuparin and xanthones, obtained from the dichloromethane extract of the leaves of *Kielmeyera coriacea* (Guttiferae) were tested against Gram-positive and Gram-negative bacteria. In addition, time-kill studies were performed to determine if aucuparin had bactericidal activity. It is not known whether the aucuparin found in *K. coriaceae* is due to *de novo* synthesis in response to diverse forms of stress, is already present in the plant, or is a combination of both. Aucuparin and 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone showed antimicrobial activities against *Bacillus subtilis* with MIC values of 3.12 µg/ml and 12.5 µg/ml, respectively, and aucuparin against *Staphylococcus aureus* with a MIC value of 12.5 µg/ml. In contrast to the relatively low MICs for Gram-positive bacteria, Gram-negative bacteria tested were not inhibited by aucuparin at concentrations ≥100 µg/ml. The kinetics of bactericidal activity were evaluated against *S. aureus* at six concentrations of aucuparin (0.25×, 0.5×, 1×, 2×, 4× and 8× the MIC). The rate of bacterial killing was dependent on the concentration of aucuparin, with more than 10^5 organisms/ml being eradicated within 8 h at the highest concentration studied. Bacterial culture was monitored for up to 24 h, and no regrowth was observed.

Keywords: *Kielmeyera coriacea*, biphenyl, xanthones, traditional medicine, antibacterial activity.

Introduction

Kielmeyera coriacea Mart. (Guttiferae), popularly know as "Pau-Santo", is a typical species found in the Brazilian Cerrado vegetation. Many plants of the Brazilian Cerrado are used as natural medicines by local populations to treat several tropical diseases including schistosomasis, leishmaniasis, malaria, fungal and bacterial infections (Alves et al., 2000).

Phytochemical investigation of the dichloromethane extracts of *K. coriacea* leaves and stems has resulted in the isolation and identification of ten xanthones, one biphenyl (aucuparin, **1**) and two triterpenes (Cortez et al., 1998). In this study, four xanthones and a biphenyl exhibited antifungal activity against the plant pathogenic fungus *Cladosporium cucumerinum*, while two prenylated xanthones inhibited the growth of *Candida albicans*.

Previous studies showed that aucuparin and its derivatives in the sapwood tissue of *Sorbus aucuparia* L. are essentially absent from healthy tissue, and are only produced as phytoalexins following fungal infection (Kokubun et al., 1995a). Phytoalexins are part of the induced chemical defence produced by plants in response to several forms of stress, including microbial attack (Bailey & Mansfield, 1982; Brooks & Watson, 1985). The possible role of the biphenyl aucuparin from *K. coriacea* as a phytoalexin is still unclear, although it may play a role in the plant's chemical and biochemical defence mechanisms.

In the present study we describe the testing of *in vitro* antibacterial activity of aucuparin and the xanthones from *K. coriacea* against Gram-positive and Gram-negative bacteria. In addition, time-kill studies were performed to determine if aucuparin had bactericidal activity.

Materials and methods

Compounds

All compounds tested in this study were isolated by Cortez et al. (1998). Briefly, *K. coriacea* was collected in Mogiguaçu

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(São Paulo, Brazil) in July 1995 and a voucher sample was deposited (no. SP298463) at the Herbarium of the State Botanical Institute, São Paulo, Brazil. Analysis of the dichloromethane extract of *K. coriacea* leaves and stems by high performance liquid chromatography coupled with a photodiode array detector (LC-UV) and thermospray mass spectrometer (TSP/LC-MS) revealed the presence of several xanthones. Phytochemical investigation of this extract resulted in the isolation and identification of ten xanthones, one biphenyl and two triterpenes. Structures were established by chemical and spectroscopic methods (UV, EI-MS, D/CI-MS, ^{1}H NMR, ^{13}C NMR, HMQC and HMBC).

Microorganisms and growth conditions

The following microorganisms were used for detecting antibacterial compounds: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* ATCC 6623 and *Staphylococcus aureus* ATCC 25923. *S. aureus* penicillin-susceptible and -resistant strains were obtained from local clinical microbiology laboratories. Cultures of these bacteria were grown in nutrient broth (Difco) at 37 °C and maintained on nutrient agar slants at 4 °C.

Reference antibiotics

The following chemotherapeutic agents (Sigma Chemical Co., St. Louis, MO, USA) were included in the test as control: vancomycin and penicillin.

Antibacterial susceptibility testing

The minimum inhibitory concentrations (MICs) of all compounds and reference antibiotics were determined by microdilution techniques in Mueller-Hinton broth (Merck) as described by the National Committee for Clinical Laboratory Standards (1997a,b). Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard (10^{8} colony-forming units [CFU]/ml) and diluted 1 : 100 for the broth microdilution procedure. Microtiter trays were incubated at 37 °C and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolate. The endpoint MIC is the lowest concentration of compounds at which the microorganism tested does not demonstrate visible growth. MBCs were defined as the lowest concentration yielding negative subcultures or only one colony.

Time-kill curve methodology

Prior to experimentation, the lower limit of bacterial quantification and the potential of compound carryover during the plating process were determined as previously described (Klepser et al., 1998). Bacterial suspensions of *S. aureus* and *B. subtilis* were prepared in sterile water from a 24 h culture plate and the resulting suspensions were adjusted to a 0.5 McFarland turbidity standard (approximately 10^{8} CFU/ml). These suspensions were then serially diluted to produce bacterial concentrations of approximately 500, 100, 50 and 30 CFU/ml for each isolate. Portions (10 µl) were then removed from each suspension and plated on Mueller-Hinton agar plates for colony count determination. The plates were incubated at 37 °C for 24 h. The lower limit of reproducibly detectable CFU per milliliter was defined as the most dilute suspension that enabled viable-colony counting with a reproducibility coefficient of variation of <25%.

For the compound carryover experiments, the standardized bacteria suspensions obtained as described above were diluted to inoculates of approximately 10^{3} CFU/ml. An aliquot (100 µl) from each bacterial suspension was then added to 900 µl of either sterile water alone or sterile water plus aucuparin from *K. coriaceae* (20 or 40 µg/ml) for a cell concentration of 10^{2} per ml. Immediately following the addition of the bacterial suspension to the aqueous solutions, portions (10 and 30 µl) were removed and streaked across Mueller-Hinton agar plates for colony count determination. Compound carryover was defined as a reduction in colony counts from a compound-bacteria suspension of >25% compared to the colony count from a control sample (no aucuparin).

The effects of aucuparin on the growth of *S. aureus* and *B. subtilis* were conducted by preparing a standardized suspension (0.5 McFarland) of each isolate as described above. A 1 : 10 dilution of these suspensions was made by adding 1 ml of bacterial suspension to 9 ml of Mueller-Hinton broth with or without the desired amount of aucuparin. The antibacterial activity of aucuparin was studied over a range of multiples of MIC encompassing 0.25 to 16× MIC. Tests were performed in triplicate and incubated at 37 °C. At predetermined time points (0, 2, 4, 6, 8, 10, 12, 16, 24, 26, 30 and 48 h) a 0.1-ml sample was removed from each test solution, serially diluted in sterile water, and plated (10 µl) on Mueller-Hinton agar plates for colony count determination. Plates were incubated at 37 °C for 24 h before colony count determination. Data from triplicate runs were averaged and plotted as logarithms of CFU per milliliter versus time for each time point.

Results and discussion

The biphenyl (**1**) and xanthones were obtained from the dichloromethane fractions of *K. coriacea* by column chromatography on silica gel and gel filtration on Sephadex LH-20 and were identified by comparison of their ^{1}H NMR, ^{13}C NMR, EI- and D/CI-MS with literature data (Cortez et al., 1998).

The results of susceptibility tests of aucuparin (**1**) and the most active xanthone [1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone (**2**)] from *K. coriacea* are presented in Table 1. Aucuparin and the xanthone showed antimicrobial activity against *B. subtilis* with MICs of 3.12 µg/ml and 12.5 µg/ml, respectively, and against *S.aureus* with a MIC of

Table 1. Minimum inhibitory concentration (MIC) of aucuparin (**1**), 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone (**2**) and reference antibiotics against a range of microorganisms.

Organism	MIC (μg/ml)			
	Aucuparin	Xanthone	Penicillin	Vancomycin
Staphylococcus aureus	12.5	12.5	0.06	NA
Escherichia coli	100	>100	NA	NA
Bacillus subtilis	3.12	12.5	NA	0.39
Pseudomonas aeruginosa	100	>100	NA	NA

NA: not assayed

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ($\mu g\,ml^{-1}$) of aucuparin (**1**) and 1,3,7-trihydroxy-2-(3-methylbut-2-enyl)-xanthone (**2**) against *S. aureus* penicillin-susceptible and -resistant strains.

Microorganisms	Aucuparin ($\mu g\,ml^{-1}$)		Xanthone ($\mu g\,ml^{-1}$)	
	MIC	MBC	MIC	MBC
Staphylococcus aureus penicillin-resistant				
011/94	6.25	12.5	25	50
517/95	6.25	12.5	25	50
100/94	6.25	12.5	25	50
Staphylococcus aureus penicillin-susceptible				
09/94	12.5	25	25	50
642/98	12.5	25	50	50

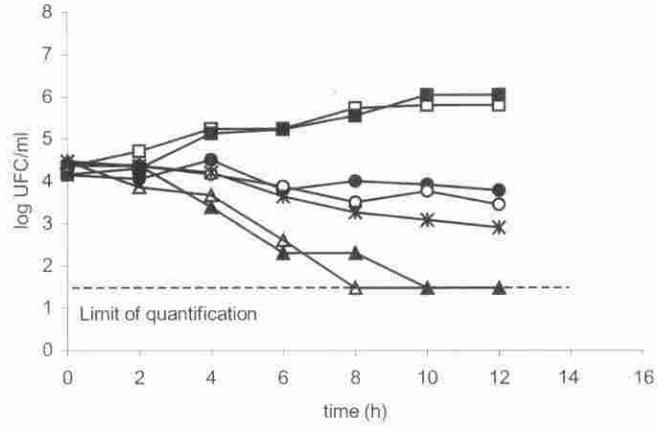

Figure 1. Time-kill of aucuparin against *S. aureus*. Symbols indicate inhibitory concentrations as follows: ■ control; □ 0.25× MIC; ● 0.5× MIC; ○ 1× MIC; ✳ 2× MIC; ▲ 4× MIC; △ 8× MIC.

12.5 μg/ml. The qualitative antimicrobial activity shown by the MIC values indicated that aucuparin and the xanthone showed significant differences among the tested microorganisms. In contrast to the relatively low MICs for Gram-positive bacteria, Gram-negative bacteria tested were not inhibited by aucuparin and the xanthone at concentrations ≥100 μg/ml. The antimicrobial activities of this xanthone were much stronger than that of other xanthones from *K. coriacea* against *S. aureus* and *B. subtilis* (data not shown).

Similar results were obtained with strains from clinical specimens. Because penicillin-resistant *Staphylococcus aureus* (PRSA) strains seemed to be more sensitive to aucuparin and xanthone than penicillin-susceptible *S. aureus* (PSSA), we compared the antibacterial activity of aucuparin and xanthone for PRSA and PRSA strains from clinical specimens. MICs and MBCs for PRSA were significantly lower than those for PSSA (Table 2). This is particularly noteworthy because the majority of penicillin-resistant staphylococci are often resistant to many other unrelated drugs.

Iinuma et al. (1996) have reported the antibacterial activity of xanthones from Guttiferaceous plants against methicillin-resistant *S. aureus* (MRSA). According to these authors, one active isolate, alpha-mangostin, had a MIC of 1.57–12.6 μg/ml. These authors showed that rubraxanthone, which was isolated from *Garcinia dioica* and has a structure similar to that of mangostin, had the highest activity against staphylococcal strains (MIC 0.31–1.25 μg/ml).

The incidence of severe nosocomial infections caused by Gram-positive cocci has increased in the last decades. This increase has been accompanied by the resistance of these microorganisms to multiple antimicrobials (Villanova et al., 1989). The recent emergence of bacterial infections and resistant strains has stimulated the development of novel antibacterial drugs.

Time-kill studies were performed to determine if aucuparin had bactericidal activity. The kinetics of bacterial killing were evaluated against *S. aureus* at six concentrations of aucuparin (0.25×, 0.5×, 1×, 2×, 4×, and 8× the MIC). No aucuparin carryover was noted with the employed methodology. The lowest reproducibly quantifiable concentration of bacteria was 50 CFU/ml. Cell viability at predetermined time points (0 to 16 h) was measured. As shown in Fig. 1, the rate of bacterial killing was dependent on the concentration of aucuparin, with greater than 3-log-unit (>10^5

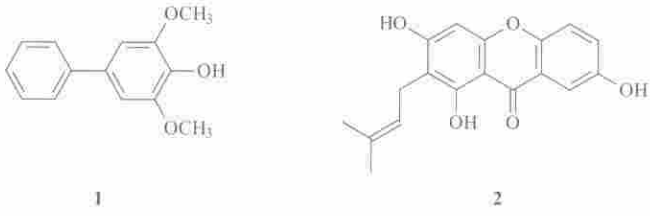

Figure 2. Structures of compounds **1** and **2**.

organisms/ml being eradicated) within 8 h with the highest concentration studied, indicating the potent bactericidal activity of the compound. Control (no-drug) exhibited a 2-log-unit increase in CFU/ml after 8 h. Bacterial cultures were monitored for up to 24 h, and no regrowth was observed. The minimum concentration of an antimicrobial necessary to kill an organism, MBC, should be equal to or greater than the MIC for that microbe. In this study, however, the concentrations 0.5×, 1×, and 2× the MIC have a similar inhibitory effect on the growth of the organisms. Time-kill data that correspond to the MIC, plus or minus one dilution, represent a transition from minimum activity (subinhibitory concentrations) to maximal antibacterial effect (suprainhibitory concentrations) and encompass the concentration that produces 50% of the maximal effect.

In the present paper, we report on the aspects of the *in vitro* activity of aucuparin (**1**) isolated from *K. coriacea*. The occurrence of aucuparin has been linked to induced chemical defenses produced by plants in response to microbial attack (Bailey & Mansfield, 1982; Brooks & Watson, 1985, Pedras & Khan, 2000; Pedras et al., 2000). Recently, the antifungal activity of medicinal plants against *Aspergillus niger* was evaluated (Iida et al, 1999). In this study, an active compound was identified as broussonin A [2-(3-(4-hydroxyphenyl)propyl)-5–methoxyphenol] which was formerly reported as a phytoalexin of *Broussonetia papyrifera*. Docherty et al. (1999) studied the inhibition of herpes simplex replication by resveratrol. These authors have shown that resveratrol, a phytoalexin, inhibited herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) replication in a dose-dependent, reversible manner. The observed reduction in virus yield was not caused by the direct inactivation of HSV by resveratrol nor inhibition of virus attachment to the cell. According to these authors, the chemical did, however, target an early event in the virus replication cycle since it was most effective when added within 1 h of cell infection, less effective if addition was delayed until 6 h post-infection and not effective if added 9 h post-infection.

Kokubun and Harborne (1994) studied phytoalexin induction in leaves of the Rosaceae by copper ions. They reported that both biotic and abiotic induction was examined and antifungal compounds were detected in 47 species. According to the authors, these compounds appeared to be constitutive metabolites, released from bound phenolic materials already present in the leaf. Aucuparin was identified as a phytoalexin from the leaves of *Sorbus aucuparia*.

More recently, Kokubun and Harborne (1995b) have reported that following fungal inoculation or natural infection, five biphenyl phytoalexins (aucuparin and its 2′ and 4′ oxygenated derivatives) were induced variously in the sapwood of *Aronia, Chaenomeles, Eriobotrya, Malus* and of *Sorbus aucuparia*. By contrast, they have shown that 14 dibenzofuram phytoalexins were induced variously in sapwood of *Cotoneaster, Crateagus, Cydonia, Mespilus, Photinia, Pseodocydonia, Pyracantha* and two *Sorbus* spp. It is not known whether the aucuparin detected in *K. coriacea* is due to *de novo* synthesis in response to diverse forms of stress, release from material already present in the plant or a combination of these.

A more extensive study *in vitro* and *in vivo* of aucuparin associated with both constitutive and induced defences of plants may be helpful in determining the potential usefulness of compounds from *K. coriacea* for the treatment of infections caused by Gram-positive bacteria.

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