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Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*

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ABSTRACT

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Aims: We investigated the antimicrobial activities of eucalyptus leaf extracts to find effective antibacterial agents.

Methods and Results: The antimicrobial activities of leaf extracts from 26 species of eucalyptus were measured. Extracts of *Eucalyptus globulus*, *E. maculata* and *E. viminalis* significantly inhibited the growth of six Gram-positive bacteria (*Staphylococcus aureus*, MRSA, *Bacillus cereus*, *Enterococcus faecalis*, *Alicyclobacillus acidoterrestris*, *Propionibacterium acnes*), and of a fungus (*Trichophyton mentagrophytes*), but they did not show strong antibacterial activity against Gram-negative bacteria (*Escherichia coli*, *Pseudomonas putida*). 2′,6′-dihydroxy-3′-methyl-4′-methoxy-dihydrochalcone, eucalyptin and 8-desmethyl-eucalyptin, isolated from *E. maculata* extracts, exhibited potent antimicrobial activities against seven micro-organisms with minimum inhibitory concentrations (MIC) ranging from 1·0 to 31 mg l^{-1}.

Conclusions: The eucalyptus extracts and three compounds from *E. maculata* were found to be effective against micro-organisms that cause food poisoning, acne and athlete's foot.

Significance and Impact of the Study: This study shows potential uses of extracts from *E. globulus*, *E. maculata* and *E. viminalis*, and antimicrobial compounds isolated from *E. maculata*.

Keywords: antimicrobial, bacteria, eucalyptus, extracts, leaf, pathogens.

INTRODUCTION

Eucalyptus is native to Australia, and the genus *Eucalyptus* contains about 600 species. Of all the species, *Eucalyptus globulus* is the most widely cultivated in subtropical and Mediterranean regions. As eucalyptus is a fast-growing tree, and is a suitable ingredient for paper manufacture, there has been extensive overseas forest plantation of eucalyptus trees. Leaves are a byproduct of tree cutting, and the use of the excess leaves for biomass resources is considered to be an important research subject.

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The Aborigines (native Australians) have traditionally used eucalyptus leaves to heal wounds and fungal infections. Leaf extracts of eucalyptus have been approved as food additives, and the extracts are also currently used in cosmetic formulations. Recently, attention has been focused on the medicinal properties of these extracts. Research data has demonstrated that the extracts exhibited various biological effects, such as antibacterial, antihyperglycemic (Gray and Flatt 1998) and antioxidant (Lee and Shibamoto 2001) activities. It has been reported that macrocarpals from *E. macrocarpa* (Yamakoshi *et al.* 1992) and grandinol from *E. perriniana* (Nakayama *et al.* 1990) were effective against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*). In this study, we investigated the antimicrobial activities of eucalyptus leaf extracts (26 species) against

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nine different species of pathogenic micro-organism that cause food poisoning, acne and athlete's foot.

MATERIALS AND METHODS

Media and chemicals

All formulated media [nutrient broth, brain–heart infusion (BHI) broth, trypticase soya broth] were obtained from Difco. The media ingredients and hinokitiol were purchased from Wako Pure Chemical Industries Ltd (Hiroshima, Japan). Trichlosan (Irgasan DP300) was obtained from Ciba Geigy (Tokyo, Japan).

Micro-organisms and culture conditions

The micro-organisms used for antimicrobial assay and culture conditions were as follows: *B. cereus* (IFO3001; Institute for Fermentation, Osaka, Japan), *Escherichia coli* (IFO15043), *Pseudomonas putida* (IFO3738), *S. aureus* (IFO12732), methicillin-resistant *S. aureus* (MRSA; RIM0310925) – nutrient broth (37°C); *Enterococcus faecalis* (IFO12970) – BHI broth (37°C); *Alicyclobacillus acidoterrestris* (ATCC49025; American Type Culture Collection) – BAM broth/ATCC1655 (50°C); *Propionibacterium acnes* (ATCC6919) – trypticase soya broth (37°C); *Trichophyton mentagrophytes* (IFO5466) – Sabouraud's broth (28°C).

Preparation of extracts

Eucalyptus leaves were obtained from the Forestry Research Institute and the Sydney office of Oji Paper Co., Ltd. The leaf extracts of eucalyptus (26 species) were prepared as follows. The leaves (10 g) were dried *in vacuo* and immersed in 200 ml methanol–dichloromethane (1 : 1) at room temperature for 2 days. The solvents were separated from the leaves by filtration and concentrated to give methanol–dichloromethane extracts.

Isolation of antimicrobial compounds

The dried leaves (500 g) of *E. maculata* were immersed in *n*-hexane (6 l) at room temperature for 2 days. The solvent

Table 1 Antimicrobial activities of methanol–dichloromethane extracts prepared from eucalyptus leaves

	MIC (mg l^{-1})								
	S. a	MRSA	B. c	E. f	A. a	P. a	E. c	P. p	T. m
E. blakelyi	31	31	31	125	>250	250	>250	>250	250
E. botryoides	31	31	31	31	>250	125	>250	31	125
E. bridgesiana	16	16	16	63	7·8	250	>250	>250	250
E. caley	31	63	63	63	7·8	63	>250	>250	63
E. camaldulensis	63	63	125	125	>250	125	>250	>250	125
E. cephalocarpa	16	16	16	31	>250	63	>250	>250	63
E. cinerea	63	63	31	63	>250	63	>250	>250	63
E. cosmophylla	16	16	7·8	16	>250	31	>250	>250	125
E. crebra	63	31	31	125	>250	125	>250	>250	125
E. dealbata	>250	>250	>250	>250	>250	>250	>250	>250	>250
E. drepanophylla	16	16	31	125	7·8	63	>250	>250	63
E. eximia	16	16	31	31	>250	31	>250	>250	125
E. globulus	3·9	3·9	7·8	31	7·8	3·9	>250	63	31
E. grandis	63	31	31	31	63	63	>250	63	250
E. intermedia	63	31	63	125	>250	250	>250	>250	250
E. maculata	3·9	3·9	7·8	31	7·8	3·9	>250	250	31
E. maidenii	16	16	16	16	7·8	63	>250	>250	63
E. melliodora	63	63	63	125	>250	125	>250	>250	125
E. microtheca	31	63	63	63	>250	125	>250	>250	125
E. nitens	>250	125	>250	>250	31	>250	>250	31	>250
E. nortonii	31	31	16	31	>250	31	>250	>250	125
E. punctata	63	63	31	63	>250	125	>250	>250	125
E. robusta	31	31	16	63	7·8	125	>250	>250	125
E. saligna	7·8	7·8	125	125	31	31	>250	>250	250
E. tereticornis	7·8	7·8	31	125	>250	>250	>250	>250	>250
E. viminalis	3·9	3·9	3·9	15·6	15·6	3·9	>250	>250	31

S. a, *Staphylococcus aureus*; B. c, *Bacillus cereus*; E. f, *Enterococcus faecalis*; A. a, *Alicyclobacillus acidoterrestris*; P. a, *Propionibacterium acnes*; E. c, *Escherichia coli*; P. p, *Pseudomonas putida*; T. m, *Trichophyton mentagrophytes*.

(hexane) was removed by filtration, and acetone (6 l) was added to the residual leaves and stored at room temperature for 3 days. The solvent (acetone) was then concentrated *in vacuo* to yield 65 g of extracts. The extracts were then partitioned with *n*-hexane and water, and the aqueous layer was consecutively partitioned with dichloromethane, ethyl acetate and *n*-butanol. The ethyl acetate fraction (active fraction) was concentrated to give 23 g of extracts. Subsequently, the extracts were loaded on a silica gel column and eluted with a stepwise gradient of hexane–ethyl acetate. The fraction with antibacterial activity (hexane–ethyl acetate, 3 : 1) was further separated by ocatadecyl silica-HPLC with 80% methanol to yield compound 1 (1·33 g), compound 2 (156 mg) and compound 3 (125 mg). Compounds 1–3 were analysed by ultraviolet (u.v.), infra-red (IR), nuclear magnetic resonance (NMR) and electrospray mass spectrometry.

Antimicrobial assay

All micro-organisms were cultured at appropriate conditions described in 'Micro-organisms and culture conditions'. All bacteria were cultured in liquid media for 4–5 h, and the concentration of bacterial suspension was adjusted to *ca* 5×10^8 CFU ml^{-1}. After growing on Sabouraud's agar for 10 days, the spores of *T. mentagrophytes* were suspended in Sabouraud's broth, and the concentration of spores was adjusted to *ca* 5×10^6 spores ml^{-1}. The test samples (extracts and purified compounds) were dissolved in dimethyl sulphoxide (DMSO), and a twofold serial dilution of each sample was prepared. A 50-μl of each sample was added to 940 μl of culture medium prior to inoculation with 10 μl of bacterial suspension or 10 μl of spore suspension. Each sample was then incubated at appropriate temperature for 48 h (bacteria) or 7 days (*T. mentagrophytes*). The minimum inhibitory concentration (MIC) values were defined as the lowest concentration of test samples that inhibited visible growth of micro-organisms. The antibacterial agents (hinokitiol and trichlosan) were dissolved in sterilized distilled water and MICs were determined according to the procedure described above.

RESULTS

Table 1 shows the antimicrobial activities of methanol–dichloromethane extracts prepared from 26 species of eucalyptus leaves. It was found that the antimicrobial activities of the extracts against nine different micro-organisms varied considerably among *Eucalyptus* spp. Of the 26 species tested, the extracts of *E. globulus*, *E. maculata* and *E. viminalis* significantly inhibited the growth of Gram-positive bacteria (*S. aureus*, MRSA, *B. cereus*, *Ent. faecalis*, *A. acidoterrestris*, *P. acnes*) and a fungus (*T. mentagrophytes*), but these extracts did not show strong antibacterial activities against Gram-negative bacteria (*E. coli*, *Ps. putida*). By contrast, the extracts of *E. botryoides* and *E. nitens* specifically inhibited the growth of both Gram-negative and Gram-positive bacteria.

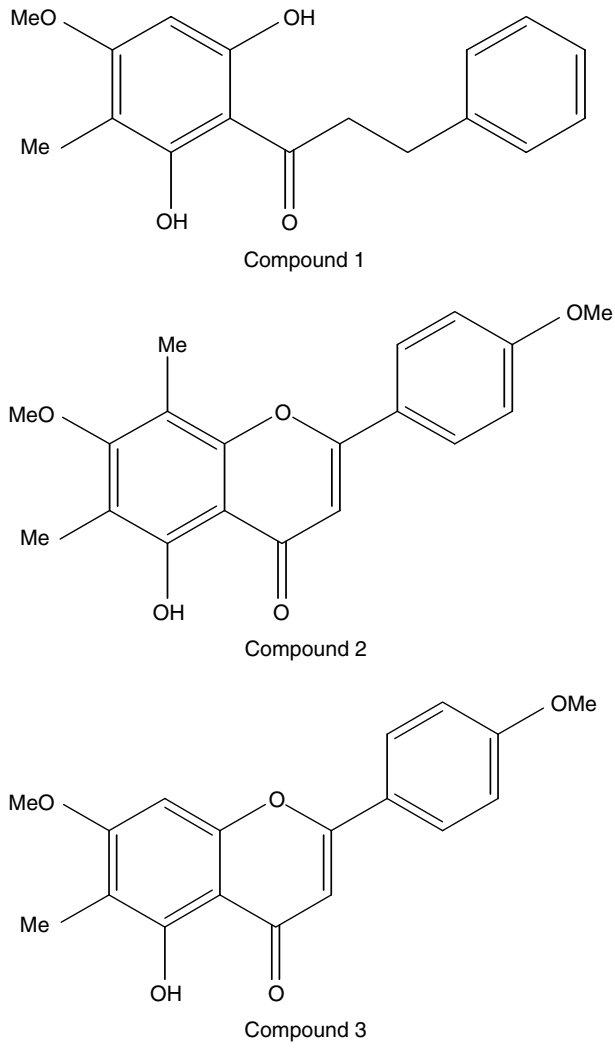

Fig. 1 Structure of antimicrobial compounds isolated from *Eucalyptus maculata*

The structures of three compounds, isolated from the leaf extracts of *E. maculata*, are shown in Fig. 1. The structures of compounds 1–3 were determined by ^{1}H- and ^{13}C-NMR spectra in conjunction with analysis of u.v. and IR spectra. Compounds 1, 2 and 3 were identified as 2′,6′-dihydroxy-3′-methyl-4′-methoxy-dihydrochalcone (Malterud 1992), eucalyptin (Lamberton 1964) and 8-desmethyl-eucalyptin (Horn *et al.* 1964), respectively.

The yields of compounds 1, 2 and 3 from *E. maculata* leaves were 0·22, 0·031 and 0·025% (w/w), respectively. HPLC data (spiking experiments with purified samples) showed that the other species such as *E. botryoides*, *E. crebra*

Table 2 Antimicrobial activities of compounds isolated from *Eucalyptus maculata*

	MIC (mg l^{-1})						
	S. a	MRSA	B. c	E. f	P. a	E. c	T. m
Compound 1	3·9	7·8	3·9	7·8	2·0	>63	1·0
Compound 2	1·0	2·0	1·0	3·9	1·0	>63	31
Compound 3	1·0	2·0	1·0	3·9	1·0	>63	31
Trichlosan	0·25	0·5	0·5	3·9	7·8	3·9	2·0
Hinokitiol	7·8	15·6	7·8	31	>125	31	>125

S. a, *Staphylococcus aureus*; B. c, *Bacillus cereus*; E. f, *Enterococcus faecalis*; P. a, *Propionibacterium acnes*; E. c, *Escherichia coli*; T. m, *Trichophyton mentagrophytes*.
Compound 1: 2′,6′-dihydroxy-3′-methyl-4′-methoxy-dihydrochalcone.
Compound 2: eucalyptin.
Compound 3: 8-desmethyl-eucalyptin.

and *E. globulus* contained these three flavonoids, and that the content in these *Eucalyptus* species was lower than that in *E. maculata* (data not shown).

As presented in Table 2, the three flavonoids commonly exhibited significant inhibitory activities against Gram-positive bacteria and a fungus (*T. mentagrophytes*), with MIC ranging from 1·0 to 31 mg l^{-1}. However, it should be noted that compound 1 was more effective against *T. mentagrophytes* and less effective against Gram-positive bacteria than compounds 2 and 3. The antimicrobial activities of these three flavonoids against Gram-positive bacteria and *T. mentagrophytes* were greater than those of hinokitiol (a natural antibacterial agent). The antibacterial activity of the three flavonoids against *P. acnes* was greater than that of trichlosan (a synthetic antibacterial agent), and the antifungal activity of compound 1 against *T. mentagrophytes* was slightly greater than that of trichlosan.

DISCUSSION

The data presented in Table 1 suggest that various antimicrobial constituents with different spectra are present in the leaves of *Eucalyptus* spp. It has been reported that trichlosan interacted with an enzyme in the fatty acid biosynthetic pathway and exhibited inhibitory activities against both Gram-positive and Gram-negative bacteria (Heath *et al.* 1998; Heath *et al.* 2000). The main difference between Gram-positive bacteria and Gram-negative bacteria is the structure of their cell walls. Unlike Gram-positive bacteria, Gram-negative bacteria have a high content of lipopolysaccharide layer in the cell wall. Therefore, it is postulated that the three flavonoids isolated from *E. maculata* are unable to pass through the lipopolysaccharide layer of Gram-negative bacteria. The inhibitory mechanism in both Gram-positive bacteria and the fungus (*T. mentagrophytes*) is considered to be an interesting subject for investigation.

For some micro-organisms, both the purified compounds and crude extracts of *E. maculata* showed the same MIC values; this suggests that the MIC values of crude extracts are associated with the ratio of antimicrobial compounds in the extracts.

We have selected *Eucalyptus* clones possessing potent antibacterial activities, and have developed antibacterial agents containing the leaf extracts. The antibacterial activity of the agent was maintained for *ca* 1 month after spreading the agent on stainless and plastic sheets (Anon. 1999). This antibacterial agent is currently used in the formulation of wet tissues and other commercial products. Finally, the eucalyptus extracts were found to be effective against pathogens causing food poisoning, acne, and athlete's foot, and a wide range of commercial applications (kitchen, restaurants, ingredients of cosmetics, sanitary products, etc.) can be anticipated.

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