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Antibacterial compounds from the Australian native plant *Eremophila glabra*

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ABSTRACT

Recent reports of *Eremophila glabra* (R.Br.) Ostenf. (Scrophulariaceae) displaying antibacterial activity has led us to investigate the bioactive secondary metabolites responsible for this activity. Bioassay-directed fractionation of solvent extracts prepared from the leaves of *E. glabra* led to the isolation of seven serrulatane diterpenes, three flavonoids and the caffeoyl ester disaccharide verbascoside. Among these, four serrulatanes, namely 18-acetoxy-8, 20-dihydroxyserrulat-14-en-19-oic acid (**14**), 18,20-diacetoxy-8-hydroxyserrulat-14-en-19-oic acid (**16**), 8,18,20-triacetoxyserrulat-14-en-19-oic acid (**17**) and 18-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**18**) are described for the first time, while 8,20-diacetoxyserrulat-14-en-19-oic acid (**3**), 8,18,20-trihydroxyserrulat-14-en-19-oic acid (**5**) and 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**19**) were previously reported. All three flavonoids hispidulin (**12**), jaceosidin (**13**) and cirsimaritin (**15**) are known but reported for the first time in *E. glabra.* All compounds were tested in an agar diffusion antimicrobial assay against *Staphylococcus aureus* (NCTC 10442) and *Staphylococcus epidermidis* (ATCC 14990). Compounds **12**, **13**, **17**, **18** and **19** exhibited moderate activity, with minimum inhibitory concentrations (MICs) ranging from 32 to 512 μg/mL. Compound **19** demonstrated the highest activity against *S. epidermidis* ATCC 14990 with MIC of 32 μg/mL, while **13** demonstrated the highest activity against *S. aureus* NCTC 10442 with MIC of 128 μg/mL.

1. Introduction

Eremophila (Scrophulariaceae) are endemic Australian native plants represented by over 200 different species [1]. Several species of *Eremophila* have been reported to be used by the Australian Aboriginal people for relieving a number of disorders such as cold, fever, influenza, insomnia, and internal pain [2]. While some species were used for treatment of rheumatism, diarrhoea, and to encourage deep sleep [3]. From a western perspective, these species have the potential to assist in the treatment of illnesses that mainly come from bacterial origin, and as such there have been reports of *Eremophila* demonstrating antimicrobial activities [3,4]. A broad antimicrobial screening program of 72 *Eremophila* species, which included *E. glabra,* showed that many of these species have antimicrobial activity against Gram-positive organisms related to some important human diseases [6].

Recently, in a study of Australian native plants that can reduce ruminal lactic acidosis, *E. glabra* was found to be the most effective plant [7]. Further investigation revealed that extractable compounds, in particular serrulatane diterpenes in this plant, were inhibitory to the lactate-producing Gram positive bacterium *Streptococcus bovis*[8]. In another study, screening of 128 Australian perennial plants showed that *E. glabra* was also one of the most active in reducing methane emissions from ruminants [9]. A follow up study using an in vitro Rusitec system further confirmed the methane reducing ability of *E. glabra* and showed that a significant reduction in methane production by 45% was linked to a reduction in methanogen populations by 42.1% compared with the control substrate [10].

From a phytochemistry perspective, *Eremophila* species are considered a rich source of novel and interesting secondary metabolites that have been well studied over the years [3]. Chemical investigation of the resin from a number of *Eremophila* species has resulted in the isolation of a large variety of novel diterpenoids [11]. Diterpenes are the major represented class in *Eremophila* species [3], and the serrulatane skeleton (**1**, Fig. 1), which is unique to *Eremophila*, is the most common diterpene encountered in this genus [12]. Ghisalberti had previously suggested that the serrulatanes might possess antimicrobial activity based on their terpenoid/phenolic structure [13]. Subsequently, an examination of the extracts of *Eremophila duttonii* for antibacterial activity showed that the serrulatane type compounds were the principal components responsible for the antibacterial activity in this plant [14]. Furthermore, serrulatanes isolated from *E. sturtii* were found to have antibacterial activity against *Staphylococcus aureus*[15] in

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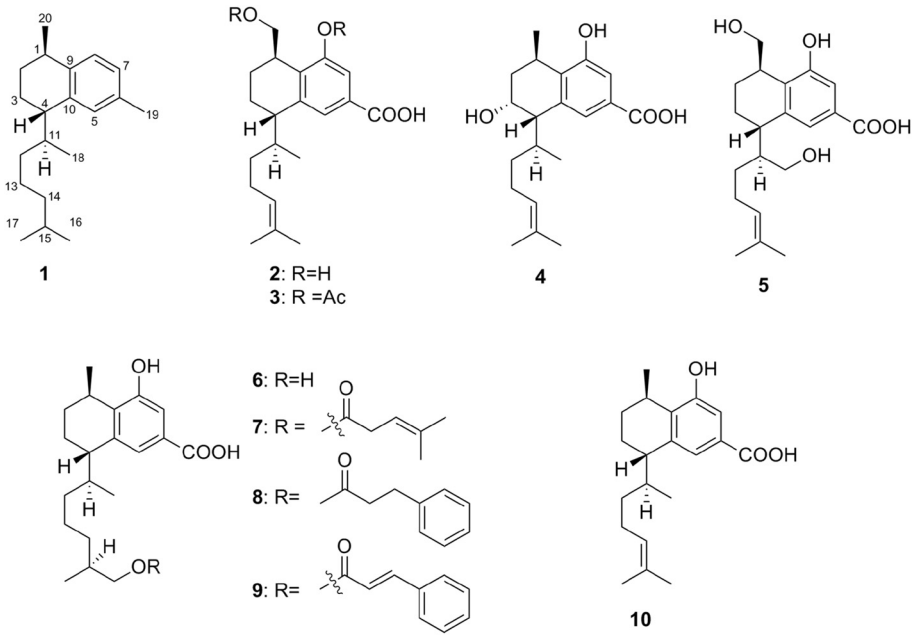

Fig. 1. General serrulatane diterpene structure along with examples of serrulatanes previously isolated from *E. glabra.*

addition to serrulatanes isolated from *E. neglecta,* which showed activity against other medically important bacteria [16,17].

Previous chemical investigations of *E. glabra* has led to the isolation of 8,20-dihydroxyserrulat-14-en-19-oic acid (**2**, Fig. 1) and the corresponding diacetate (**3**) as the major serrulatanes produced by this plant [12]. In addition to **2** and **3**, the isolation of 3,8-dihydroxyserrulat-14-en-19-oic acid (**4**) and 8,18,20-trihydroxyserrulat-14-en-19-oic acid (**5**) from *E. glabra* has also been reported. Interestingly, **2–5** along with other serrulatane diterpenes isolated from *E. rugosa* and *E. denticulata,* were shown to inhibit ruminal lactic-acid forming bacteria to varying degrees (**4** essentially not active, **5** the most active) [8]. More recently, five additional serrulatanes have been isolated from *E. glabra* and reported to have an anti-diabetic effect, including 8,16-dihydroxyserrulat-19-oic acid (**6**), 8-hydroxy-16-[4-methylpent-3-enoyloxy]serrulat-19-oic acid (**7**), 8-hydroxy-16-hydrocinnamoyloxyserrulat-19-oic acid (**8**), 8-hydroxy-16-cinnamoyloxyserrulat-19-oic acid (**9**) [18]. In addition, 8-hydroxyserrulat-14- en-19-oic acid (**10**) was also isolated but showed no activity in this assay [18].

In this paper, we report the bioassay-guided isolation of secondary metabolites from *E. glabra* that can inhibit Gram-positive bacteria of medical significance – namely pathogenic *Staphylococcus aureus* NCTC 10442 and a commensal *Staphylococcus epidermidis* ATCC 14990. The isolation, structural elucidation, and antimicrobial evaluation of seven serrulatane diterpenes, three flavonoids and verbascoside are described herein.

2. Materials and methods

2.1. General experimental

Rapid silica filtration (RSF) was conducted using a sintered glass column (4.5 × 12 cm) containing silica gel (10 g, Grace-Davisil, 40–63 μm) equilibrated with hexanes. Flash chromatography was conducted using a thick walled glass column (4.5 × 45 cm) packed with silica gel and eluted under positive air pressure. Scaled up silica chromatography was conducted using the Reveleris X2 chromatography system equipped with a cartridge containing silica gel as the stationary phase (120 g, 40 μm, Reveleris, p/n 145,146,134). High performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC system, equipped with a photodiode array detector (PDA) and fraction collector. Analytical separations were achieved using an Apollo C_{18} Column (250 mm × 4.6 mm i.d., 5 μm, Grace-Davison) while semi-preparative HPLC separations were achieved using either an Apollo C_{18} Column (250 mm × 10.0 mm i.d., 5 μm, Grace-Davison) or Apollo C_{18} Column (250 mm × 22 mm i.d., 5 μm, Grace-Davison). UV absorbance was routinely measured at wavelengths of 220, 254 and 280 nm. NMR spectra were acquired on a Bruker Avance 600 MHz spectrometer and chemical shifts were calibrated to resonances attributed to residual solvents. High and low resolution mass spectra were obtained on a Waters Alliance e2695 HPLC connected to a Waters LCT Premier XE time-of-flight (TOF) mass spectrometer with an atmospheric pressure chemical ionization source (APCI) in positive ionization mode. Polarimetry was acquired on a Kruss Optronic P-8000 polarimeter. Solvents were of HPLC grade unless otherwise stated.

2.2. Plant material

E. glabra accession SA 45599 was grown at the UWA Future Farm Ridgefield in Pingelly (Western Australia). Plant material (leaves and stems < 5 mm) were harvested in May 2014 from multiple plants at the post flowering stage. The plant material was freeze-dried and ground through a 1.0 mm screen before use.

2.3. Antimicrobial assay

2.3.1. Bacterial strains

Reference strains for antibacterial screening were *Escherichia coli* ATCC 25922¸ *Serratia marcescens* NCTC 1377, *Staphylococcus aureus* NCTC 10442, and *Staphylococcus epidermidis* ATCC 14990. Strains were obtained from the culture collection of the School of Biomedical Sciences at The University of Western Australia. Cultures were maintained on blood agar and were stored at 4 °C until required for testing.

2.3.2. Agar- well diffusion assay

An agar-well diffusion assay based on the method published by the Clinical and Laboratory Standards Institute was used to detect activity [19]. Briefly, inocula were prepared by culturing a loopful of bacteria on a fresh blood agar plate overnight at 37 °C and then suspending bacterial growth in saline (8.5 g/L) to a concentration of approximately 5×10^8 colony forming units (CFU) per mL. This suspension was used to surface inoculate Mueller-Hinton agar plates, into which wells of 8 mm diameter were cut with a sterile cork-borer. All samples (extracts,