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Antibacterial sideroxylonals and loxophlebal A from *Eucalyptus loxophleba* foliage ☆

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ABSTRACT

Loxophlebal A, a new antibacterial formylated phloroglucinol was isolated from the mother liquor obtained after separation of sideroxylonals from the chloroform–methanol extract of leaves of *Eucalyptus loxophleba* ssp *lissophloia*. The structure of loxophlebal A was determined to be 3-desformyl sideroxylonal A by spectroscopic methods including 1D- and 2D-NMR. The stereochemistry of loxophlebal A was determined by chemical correlation with sideroxylonal A. This article also reports an efficient, simple and economic method for large scale isolation of sideroxylonals in a purity of >90% from the leaves of *Eucalyptus loxophleba* ssp *lissophloia*.

1. Introduction

The genus *Eucalyptus* is native to Australia and is cultivated mainly for timber, pulp and essential oils that have therapeutic use in pulmonary infections. *Eucalyptus* has been a rich source of bioactive secondary metabolites [1]. Robustadials are anti-malarial compounds isolated from *E. robusta* [2]. A number of euglobals have been isolated from different species of *Eucalyptus* as Epstein Barr virus inhibitors [3]. Recently, we have found that euglobals exhibit anti-leishmanial and anti-microbial activities [4]. Macrocarpals have shown activity against periodontopathic bacteria and also inhibit HIV reverse transcriptase [5–7]. Jensenone, a monomeric phloroglucinol isolated from *Eucalyptus jensenii*, is an anti-feedant compound against vertebrate herbivores [8,9]. Sideroxylonals A (**1**), B (**2**) and C (**3**) (Fig. 1) are dimeric phloroglucinol compounds found in the leaves and flower buds of some species of *Eucalyptus* [10–12]. Sideroxylonals differ from each other in stereochemistry at C-7, C-10′ and C-7′. Satoh et al. reported first isolation of sideroxylonals A and B from *Eucalyptus sideroxylon* in 0.0012% and 0.009% yield, respectively, by repeated chromatography on silica gel, sephadex LH-20 and reverse phase HPLC. Both **1** and **2** showed antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* and *Bacilus subtilis* at 3.9 and 7.8 μg/disc respectively [10]. Later studies showed sideroxylonal A to be potent marine anti-fouling agent with activity comparable to the most active compound 2,5,6-tribromo-1-methyl-gramine [11]. The effects of various formylated phloroglucinol compounds especially sideroxylonals on feeding behavior of folivorous marsupials including common ring tail possums and koalas have been extensively studied [13,14]. Although, isolation of sideroxylonals has been reported in the literature using repeated chromatography on different stationary

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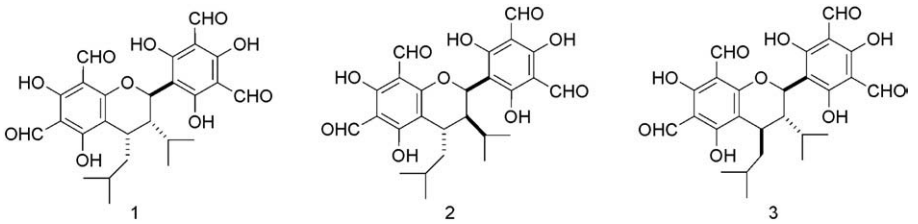

Fig. 1. Structures of sideroxylonals A, B and C.

phases with varying yields but none of these methods has potential for large scale isolation. Quantitation of sideroxylonals in *E. melliodora* and some other eucalypts by HPLC has also been reported [15,16].

As a part of our continuing program to explore biological potential of naturally occurring phloroglucinol compounds and a need of sideroxylonals in large amounts for feeding experiments on koalas and other marsupial herbivores, we planned to develop a simple and efficient method for large scale isolation of sideroxylonals from *Eucalyptus loxophleba* foliage. In this article, we report HPLC quantitation of sideroxylonals in various extracts prepared from *Eucalyptus loxophleba* ssp *lissophloia* leaves and a simple and rapid method for large scale isolation together with isolation, structure elucidation and antibacterial activity of a new dimeric phloroglucinol loxophlebal A (**4**) (Fig. 2) from the mother liquor obtained after precipitation of sideroxylonals.

2. Experimental

2.1. General

All the solvents used for extraction were of analytical grade. HPLC grade acetonitrile and methanol were used for sample preparation and in HPLC mobile phases. All chromatographic purifications were performed with silica gel #60–120 and silica gel G whereas all TLC (silica gel) development was performed on silica gel coated (Merck Kieselgel 60 F_{254}, 0.2 mm thickness) plates.

Plant material was collected form a provenance trial of *E. loxophleba lissophloia* growing at Toolbin Western Australia and managed by the Western Australian Department of Environment and Conservation. A voucher specimen has been deposited in the Gauba Herbarium at the Australian National University (WJF 09/03). The air-dried leaves of *E. loxophleba* were sent to India and were extracted by soxhlet extractor (Perfit India Ltd). Extracts were concentrated using vacuum rotary evaporator (Buchi R-114, Switzerland). IR spectra were collected on a FT-IR spectrometer (Nicolet, U.S.A.). Low resolution and high resolution electrospray ionization mass spectra were acquired on a Bruker Apex 3 instrument in both positive and negative ionization modes. ^{1}H NMR spectra were recorded on 400 MHz and ^{13}C NMR were recorded 100 MHz spectrometer (Bruker). The HPLC analysis was carried out on Kromasil (Phenomenex) C_{18} column (250×4.6 mm) connected to a Shimadzu HPLC system. Princeton SPHER-C_{18} column (250×10 mm) was used for isolation of compounds.

2.2. Sample extraction

Dried leaves of *E. loxophleba* were powdered and extracted by three methods using different solvents *viz*. hexane, chloroform, dichloromethane, ethyl acetate, diethyl ether, acetone, methanol, ethanol, acetonitrile, acetonitrile:water (6:4), ethanol:hexane (4:1), hexane:acetone (4:1) and chloroform: methanol (4:1).

2.2.1. Sonication

Sonication was performed using an ultrasonic bath (Power Sonic 510, Branson). 20 g of dried and powdered leaves was placed in conical flasks containing 60 mL solvent and sonicated for 30 min. The extract was filtered and filtrate was concentrated to dryness.

2.2.2. Maceration

20 g of dried and powdered leaves was taken in conical flasks containing 60 mL solvent and kept at 25 °C for 48 h with shaking at regular intervals. The extract was filtered and filtrate was concentrated to dryness.

2.2.3. Soxhlet extraction

For soxhlet extraction, 20 g of dried and powdered leaves was packed in a soxhlet thimble. The apparatus was fitted with a 100 mL round bottom flask containing 60 mL of solvent. The flask was heated to the reflux temperature for 48 h. After reflux, the solvent was evaporated to dryness.

2.3. HPLC analysis

The HPLC analysis was carried out on Phenomenex C_{18} column (250×4.6 mm) connected to a Shimadzu HPLC system consisting of a model LC-10AT VP fitted with 20 μL injection loop and a model, SPD-M10A VP photodiode array detector. The analysis was carried out using acetonitrile: water: TFA (93:7:0.1) as a mobile phase with a flow rate of 0.75 mL/min and run time of 20 min. The typical operating pressure was 780 psi. Class-VP software (Shimadzu) was used for both data collection and integration. Sideroxylonal A, C and B were detected at 275 nm and eluted at 12.95, 13.35 and 16.80 min, respectively. Quantitative determination was made from the calibration curve of sideroxylonal A. All the analyses were done in triplicate.

2.4. Large scale isolation of sideroxylonals

The sideroxylonal rich extract was prepared by hot soxhlet extraction using chloroform: methanol (80:20) as

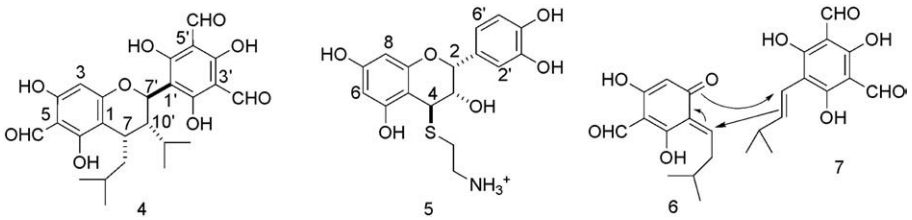

Fig. 2. Structure of loxophlebal A (**4**), 4-(2-Aminoethylthio) epicatechin (**5**) and presumed biogenetic pathway for **4**.

solvent. Powdered plant material (1.2 kg) was extracted with 20% methanol in chloroform (2.5 L) using soxhlet extractor for 48 h. After completion of extraction, solvent was evaporated to dryness on rotary evaporator to obtain 288 g of crude extract. This extract was called as sideroxylonal rich extract (SRE) and the following two methods were used to identify the best method for further purification.

2.4.1. Column chromatography

10 g of SRE was loaded on a silica gel (#60–120) column (50×7 cm). The column was eluted with 50% ethyl acetate in hexane (2.5 L, 25 fractions 100 mL each), followed by chloroform (1 L, 10 fractions 100 mL each) and finally with chloroform:methanol:acetic acid (CMA) = 95:5:0.5 (1 L, 10 fractions 100 mL each). Fractions eluted with CMA were pooled and concentrated to dryness. Dried sideroxylonal mixture was washed with methanol (10 mL×3) resulting in 0.8 g of buff white amorphous powder.

2.4.2. Selective precipitation followed by Vacuum Liquid Chromatography (VLC)

The dried SRE (10 g) was treated with methanol (50 mL), the SRE partly solubilised and the resulting solution was decanted. The residue left was then treated similarly three more times with methanol (50 mL each) after which, all of the SRE was solubilised. The resulting solutions were pooled and kept at room temperature for 30 min resulting in formation of precipitate which was allowed to settle. Mother liquor was decanted and the precipitate was further washed with methanol (20 mL×3) to obtain 0.75 g of buff white amorphous powder. This powder was subjected to vacuum liquid chromatography over silica gel G using hexane–ethyl acetate as eluent. Waxy impurities were eluted with 0–10% ethyl acetate in hexane. The column was finally eluted with 50% ethyl acetate in hexane to yield 0.38 g of mixture of three sideroxylonals.

2.4.3. Large scale processing

The above method (Section 2.4.2) was repeated on large scale with 2.7 kg of dried SRE (prepared from 10 kg dried leaves) to yield 180 g of mixture of three sideroxylonals containing waxy impurities. 50 g of this mixture was subjected to VLC on G-4 sintered glass funnel packed with TLC grade silica gel G (600 g, 13 cm I.D, column bed 8 cm). Precipitate of sideroxylonal mixture was loaded as such on the VLC column without dissolving and eluted with hexane (500 mL×10), 10% ethyl acetate in hexane (500 mL×10), and 50% ethyl acetate in hexane (500 mL×5). Finally the column was washed with ethyl acetate (2 L). Sideroxylonals were eluted in 50% ethyl acetate and the final washings to yield 22 g (>90% purity).

2.5. Isolation and purification of loxophlebal A (4)

The mother liquor left after the isolation of sideroxylonals was used for further phytochemical investigation. 100 g of mother liquor was subjected to vacuum liquid chromatography on silica gel G using hexane–ethyl acetate and then chloroform–methanol gradients. The fraction eluted in 70% ethyl acetate was re-chromatographed on silica gel #60–120 using hexane–ethyl acetate gradient. The purified fraction (300 mg) thus obtained was subjected to semi-preparative HPLC on a C_{18} column using 97% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Loxophlebal A (80 mg) was obtained as light brown solid, mp 194–196 °C, IR ν_{max} (Neat)/cm^{-1} 3435 (OH), 1635 (CHO). ^{1}H NMR (400 MHz, $CDCl_3$+ CD_3OD, 4:1) 10.12 (1H, s), 10.03 (1H, s), 9.77 (1H, s), 5.97 (1H, d, J = 11.4 Hz), 5.79 (0.6 H, brs), 3.38 (1H, m), 2.37 (1H, m), 1.76 (1H, m), 1.45 (1H, m), 1.07 (3H, d, J = 4.8 Hz), 1.02 (3H, d, J = 6.4 Hz), 0.87 (3H, d, J = 5.6 Hz), 0.73 (3H, d, J = 6.4 Hz). ^{13}C NMR (100 MHz, $CDCl_3$+ CD_3OD, 4:1) 195.9, 195.1, 194.7, 170.9, 170.0, 169.2, 166.6, 112.5, 108.7, 107.4, 106.4, 78.5, 46.5, 41.7, 32.7, 31.9, 30.9, 28.6, 27.5, 24.8, 24.5, 23.8. APCI-MS m/z 473 $[M+H]^+$, 495 $[M+Na]^+$.

2.6. Formylation of loxophlebal A (4)

A solution of loxophlebal A (10 mg) in ethyl acetate (5 mL) was stirred with DMF (5 equivalents) for 5 min. To this solution was added $POCl_3$ (5 equivalents) and the resulting solution was stirred for 30 min at room temperature. The reaction mixture was diluted with water and extracted three times with EtOAc. The organic extracts were pooled, dried over anhydrous Na_2SO_4 and concentrated. The resulting material was dissolved in MeOH and subjected to HPLC under conditions described above for sideroxylonals.

2.7. In vitro antibacterial activity

Sideroxylonals and loxophlebal A were evaluated for their *in vitro* antibacterial activity against *E. coli*, *P. aeruginosa*, *S. epidermis* and *S. aeureus*. Antimicrobial susceptibility testing was done as per National Committee for Clinical Laboratory Standards (NCCLS) microdilution assay format. Briefly, the bacterial strains were grown in prescribed media until exponential growth was achieved. Tests were performed in a 96-well microtiter plate in a final volume of 100 μL. Test compounds were dissolved in 5% DMSO at an initial concentration of 0.1 mg/mL and serially diluted in plate. Each

well was then inoculated with $2–5 \times 10^5$ bacterial cells and incubated at 37 °C for 24 h. One well containing cells and 5% DMSO without any test compound (growth control), and one well containing only growth medium (sterility control) were used as controls. Growth of bacteria was determined using Power wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). Percent survival was calculated using growth without any compound as 100% survival.

3. Results and discussion

3.1. HPLC quantitation of sideroxylonals in different extracts of *E. loxophleba*

In order to develop the best extraction conditions for efficient isolation of sideroxylonals from *Eucalyptus loxophleba* foliage, preliminary extractions were done using different extraction procedures *viz.* sonication, maceration and soxhlet extraction using different solvents *viz.* hexane, chloroform, dichloromethane, ethyl acetate, diethyl ether, methanol, ethanol, acetonitrile, acetonitrile–water, acetone–hexane, ethanol–hexane and chloroform–methanol. Analysis of all samples by HPLC revealed that soxhlet extraction was more efficient as compared with the other two methods and extraction with 20% methanol in chloroform gave maximum % yield of the extract as well as sideroxylonals. Although the amount of total sideroxylonals in DCM extract was found to be the greatest, the lower amount of material extracted using DCM (12.7 g/100 g dry leaves) indicated that the total sideroxylonals extractable by DCM extraction was much lower compared to that found using $CHCl_3$–MeOH because that solvent extracted more material overall (26.55 g/100 g dry leaves). The results are summarized in Table 1.

3.2. Isolation of sideroxylonals from Eucalyptus loxophleba foliage

For large scale isolation, the sideroxylonal rich extract (SRE) was prepared by hot soxhlet extraction using 20% methanol in chloroform as the solvent. Purification of the SRE was performed by two different methods. The first method involved silica gel column chromatography of the SRE; the polar fractions were pooled and concentrated to dryness to yield a slightly green colored powder. This was then washed with methanol in order to remove the coloring pigments which finally resulted in a buff white amorphous powder in a yield of 1.9% from the dry leaves (HPLC purity 98%).

In the second method, methanol was added in portions to the dried SRE to allow the compounds of interest to precipitate from the extract. The precipitates were allowed to settle and the solvent was decanted. These precipitates were then washed 3 times with methanol to obtain a buff white amorphous powder in a yield of 1.8% (HPLC purity 54%). This crude product was further purified by vacuum liquid chromatography. Waxy impurities were removed by initial elution with 0–10% ethyl acetate and sideroxylonals were eluted with 50% ethyl acetate in hexane. A mixture of sideroxylonals was obtained in a yield of 0.8% in a purity of more than 90% (HPLC).

Table 1
Quantitation of sideroxylonals in various extracts of *Eucalyptus loxophleba* by HPLC.

S. no.	Extract	Sideroxylonal A (%)			Sideroxylonal B (%)			Sideroxylonal C (%)			Total sideroxylonal content in extract		
		Son [a]	Mac [b]	Sox [c]	Son	Mac	Sox	Son	Mac	Sox	Son	Mac	Sox
1	Hexane	2.02 ± 0.04	0.75 ± 0.06	1.31 ± 0.03	0.65 ± 0.31	0.54 ± 0.05	0.56 ± 0.01	0.64 ± 0.40	1.36 ± 0.07	1.09 ± 0.06	3.31	2.65	2.78
2	Chloroform	6.65 ± 0.21	7.58 ± 0.40	8.85 ± 0.13	1.66 ± 0.30	1.05 ± 0.08	1.34 ± 0.04	1.48 ± 0.38	2.98 ± 0.09	3.19 ± 0.21	9.79	11.61	13.38
3	Dichloromethane	4.83 ± 0.09	5.73 ± 0.11	10.53 ± 0.42	1.12 ± 0.46	0.67 ± 0.03	1.40 ± 0.11	1.44 ± 0.43	2.05 ± 0.06	3.67 ± 0.22	7.39	8.45	15.60
4	Ethyl acetate	4.46 ± 0.20	3.62 ± 0.35	3.05 ± 0.07	1.06 ± 0.44	0.52 ± 0.01	0.88 ± 0.07	1.03 ± 0.57	1.26 ± 0.03	0.53 ± 0.05	6.55	5.40	4.46
5	Diethyl ether	5.17 ± 0.07	6.67 ± 0.07	4.78 ± 0.07	1.05 ± 0.47	0.71 ± 0.04	0.73 ± 0.07	1.19 ± 0.54	2.18 ± 0.15	1.27 ± 0.02	7.41	9.56	6.78
6	Acetone	2.56 ± 0.04	4.03 ± 0.11	3.67 ± 0.09	0.62 ± 0.10	1.00 ± 0.08	1.81 ± 0.06	0.40 ± 0.02	0.65 ± 0.09	0.83 ± 0.06	3.58	5.68	6.31
7	Methanol	5.87 ± 0.46	6.12 ± 0.20	3.11 ± 0.09	1.15 ± 0.20	1.23 ± 0.03	0.96 ± 0.07	1.41 ± 0.51	1.24 ± 0.05	1.05 ± 0.08	8.43	8.59	5.12
8	Ethanol	2.82 ± 0.24	6.06 ± 0.10	2.80 ± 0.05	0.56 ± 0.12	0.98 ± 0.03	1.61 ± 0.03	0.74 ± 0.23	1.63 ± 0.02	1.47 ± 0.07	4.12	8.67	5.88
9	Acetonitrile	4.62 ± 0.22	6.02 ± 0.14	2.54 ± 0.13	1.06 ± 0.34	1.63 ± 0.03	2.31 ± 0.14	1.02 ± 0.46	1.05 ± 0.03	1.31 ± 0.12	6.70	8.70	6.16
10	Acetonitrile: water (6:4)	3.81 ± 0.41	6.62 ± 0.01	2.04 ± 0.19	0.74 ± 0.06	2.32 ± 0.07	1.80 ± 0.07	1.01 ± 0.31	1.58 ± 0.20	0.92 ± 0.05	5.56	10.52	4.76
11	Hexane: acetone (4:1)	5.20 ± 0.29	2.08 ± 0.03	4.62 ± 0.10	1.08 ± 0.38	0.32 ± 0.04	4.33 ± 0.04	1.39 ± 0.44	0.73 ± 0.04	2.24 ± 0.20	7.67	3.13	11.19
12	Ethanol: hexane (4:1)	3.70 ± 0.17	3.70 ± 0.17	3.77 ± 0.30	0.74 ± 0.14	0.90 ± 0.04	1.05 ± 0.19	0.97 ± 0.23	1.20 ± 0.04	0.50 ± 0.01	5.41	5.80	5.32
13	Chloroform: methanol (4:1)	2.92 ± 0.32	–	7.98 ± 0.22	0.34 ± 0.03	–	2.27 ± 0.09	1.13 ± 0.10	–	1.68 ± 0.04	4.39	–	11.93

Results are expressed as Mean ± S.D. ($n = 3$).
[a] Son: sonication.
[b] Mac: maceration.
[c] Sox: soxhlet extraction.

3.3. Isolation of loxophlebal A

The mother liquor (SRE-ML) was obtained after separation of large amounts of sideroxylonals A, B, and C from the chloroform–methanol (8:2) extract of the leaves. This SRE-ML was subjected to vacuum liquid chromatography on silica gel followed by repeated column chromatography on silica gel and HPLC on reverse phase silica gel to yield loxophlebal A (**4**) (Fig. 2) as a light brown powder in a yield of 0.024%. The ESI +ve of **4** showed $[M+H]^+$ at *m/z* 473 and a relatively intense $[M+Na]^+$ at *m/z* 495 while the ESI −ve showed $[M-H]^-$ at *m/z* 471 suggesting the molecular weight of the compound to be 472. Accurate mass measurements on $[M-H]^-$, $[M+H]^+$ and $[M+Na]^+$ all suggested the common molecular formula of $C_{25}H_{28}O_9$. A characteristic fragment peak at *m/z* 251 $[C_{13}H_{15}O_5]^+$ in positive mode was also present that further fragmented with loss of a $[C_4H_7]^+$ fragment to give another ion at *m/z* 195 $[C_9H_7O_5]^+$, both characteristic for formylated phloroglucinols. The spectroscopic data indicated structural similarity of loxophlebal A to sideroxylonals.

^{1}H NMR spectrum [δ 10.12 (1H, s), 10.03 (1H, s) and 9.77 (1H, s)] and ^{13}C NMR spectrum [δ 195.9, 195.1 and 194.7] showed the presence of three formyl groups. The presence of hydrogen bonded carbonyl groups was also apparent from the IR bands at 1635 cm^{-1}. The high field region of ^{1}H NMR spectrum of **4** exhibited three methine protons at δ 3.38 (1H, m), 2.37 (1H, m) and 1.76 (1H, m); four methyl signals as doublets at δ 1.07, 1.02, 0.87 and 0.73 (3H each), suggesting two isopropyl groups. Another doublet at δ 5.97 (1H, d, J = 11.4 Hz) was typical of sideroxylonals and could be ascribed to oxymethine group at C-7′. A shoulder peak with an integral of 0.6 was present at δ 5.79 which merged with the doublet at δ 5.97 when solvent was changed to $CDCl_3$ from $CDCl_3$–CD_3OD (4:1). Oxymethine proton at δ 5.97 showed HMBC correlations with carbons at δ 106.4 (C-1′), 41.7 (C-10′) and 31.9 (C-7). Similarly benzylic proton H-7 at δ 3.38 showed HMBC correlations with carbons at δ 41.7 (C-10′), 78.5 (C-7′), 112.5 (C-1), 166.6 (C-2) and 169.2 (C-6). These correlations established the basic carbon framework of sideroxylonals.

The information from ^{1}H NMR showing only three formyl groups and MS data that gave $[M+H]^+$ peak at *m/z* 473 indicating that loxophlebal A is a desformyl sideroxylonal i.e. sideroxylonal minus CO. The proton that replaced one formyl group appeared as a broad signal at δ 5.79 integrating for 0.6 H. However, no HMBC correlations from this aromatic proton to adjacent carbons were observed making it difficult to establish the position of this aromatic proton on ring A. The broadness of aromatic proton signal in ^{1}H NMR suggested that it may be involved in some dynamic behavior (H/D exchange) or has a fast T2 relaxation and hence is likely to lose magnetization or change frequency during the course of magnetization.

Similar behavior has been reported earlier for cysteinyl-flavan-3-ol conjugates like 4-(2-aminoethylthio) epicatechin (**5**) (Fig. 2) [17]. The aromatic proton at positions 6 and 8 of flavanol showed H/D exchange and lower than normal integration in ^{1}H NMR spectra. The ^{13}C NMR spectrum also showed very low intensity signals characteristic of mono-deuterated carbons at C6 and C8. The positions 3 and 5 in loxophlebal A are in a similar environment and lead to similar conclusions. These results indicated that **4** might be either C3 or C5 desformyl sideroxylonal.

Table 2
Antimicrobial activity of sideroxylonals A, B, C and loxophlebal A.

Extract/Compound	IC_{50} (μg/mL)			
	E. coli	*P. aeruginosa*	*S. epidermis*	*S. aeureus*
SRE	>100	>12	25	25
SRE-ML	na	>25	50	>50
Sideroxylonal A	na	<25	na	na
Sideroxylonal B	na	na	na	na
Sideroxylonal C	>100	25	>50	>12
Loxophlebal A	>12	<6	>6	<3
Tetracycline (standard)	0.09	0.06	0.08	0.12

SRE = sideroxylonal rich extract, SRE-ML = sideroxylonal rich extract-mother liquor, na = not active.

Finally, in order to ascertain the location of aromatic proton, a survey of literature for ^{1}H and ^{13}C NMR shifts of related flavanols indicate that the proton at C-8 appears around δ 5.7 whereas the proton at C-6 appears downfield around δ 6.0 [18]. This indicated that **4** is 3-desformyl sideroxylonal.

Relative stereochemistry of **4** was determined by magnitude of spin coupling constant (J = 11.4 Hz) between H-7′ and H-10′ suggesting a *trans* relationship between these protons. Stereochemistry was confirmed by formylation of **4** using the Vilsmeir–Haack reagent to obtain a tetra-formylated product. It was expected that one of the three known sideroxylonals would be obtained after formylation of **4** thus confirming the stereochemistry of loxophlebal A. Reaction of **4** with phosphorus oxychloride in the presence of dimethyl formamide yielded sideroxylonal A that was identified by HPLC retention time and spiking with an authentic sample of sideroxylonal A. This indicated that loxophlebal A has the same relative stereochemistry as sideroxylonal A, i.e. protons H-7′ and H-10′ are *trans* while H-7 and H-10′ are *cis*.

Biogenetically, loxophlebal A may be formed by the Diels–Alder cyclo-addition of *O*-quinone methide (**6**) and styrene (**7**), both of which may be derived from monomeric formylated phloroglucinols present in several *Eucalyptus* species. Similar biogenetic pathways have been earlier proposed for dimeric phloroglucinols like sideroxylonals and grandinal [19,20].

3.4. Biological activity

Loxophlebal A (**4**), along with three sideroxylonals (**1**–**3**) was evaluated for its antibacterial activity against Gram-positive and Gram-negative bacterial strains. The results of *in vitro* antibacterial activity of sideroxylonals A, B and C and loxophlebal A are summarized in Table 2. Loxophlebal A showed better antibacterial activity compared with any of the sideroxylonals against the tested strains. It seems that the removal of formyl group from position 3 of sideroxylonal nucleus leads to increase in antibacterial activity against both Gram-positive and Gram-negative strains.

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