

Article

## Structures of New Phenolics Isolated from Licorice, and the Effectiveness of Licorice Phenolics on Vancomycin-Resistant *Enterococci*

Eerdunbayaer<sup>1</sup>, Mohamed A. A. Orabi<sup>1,2</sup>, Hiroe Aoyama<sup>1</sup>, Teruo Kuroda<sup>3</sup> and Tsutomu Hatano<sup>1,\*</sup>

<sup>1</sup> Department of Natural Product Chemistry, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; E-Mails: eerdun3@163.com (E.); m\_a\_orabi@yahoo.com (M.A.A.O.); ph20101@s.okayama-u.ac.jp (H.A.)

<sup>2</sup> Faculty of Pharmacy, Al-Azhar University, Assiut 71524, Egypt

<sup>3</sup> Drug Discovery and Technology Center, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; E-Mail: tkuroda@cc.okayama-u.ac.jp

\* Author to whom correspondence should be addressed; E-Mail: hatano@pharm.okayama-u.ac.jp; Tel.: +81-86-251-7936; Fax: +81-86-251-7926.

Received: 19 June 2014; in revised form: 19 August 2014 / Accepted: 20 August 2014 /

Published: 25 August 2014

---

**Abstract:** Licorice, which is the underground part of *Glycyrrhiza* species, has been used widely in Asian and Western countries as a traditional medicine and as a food additive. Our continuous investigation on the constituents of roots and stolons of *Glycyrrhiza uralensis* led to the isolation of two new phenolics, in addition to 14 known compounds. Structural studies including spectroscopic and simple chemical derivatizations revealed that both of the new compounds had 2-aryl-3-methylbenzofuran structures. An examination of the effectiveness of licorice phenolics obtained in this study on vancomycin-resistant strains *Enterococcus faecium* FN-1 and *Enterococcus faecalis* NCTC12201 revealed that licoricidin showed the most potent antibacterial effects against both of *E. faecalis* and *E. faecium* with a minimum inhibitory concentration (MIC) of  $1.9 \times 10^{-5}$  M. 8-( $\gamma,\gamma$ -Dimethylallyl)-wighteone, isoangustone A, 3'-( $\gamma,\gamma$ -dimethylallyl)-kievitone, glyasperin C, and one of the new 3-methyl-2-phenylbenzofuran named neoglycybenzofuran also showed potent anti-vancomycin-resistant *Enterococci* effects (MIC  $1.9 \times 10^{-5}$ – $4.5 \times 10^{-5}$  M for *E. faecium* and *E. faecalis*). The HPLC condition for simultaneous detection of

the phenolics in the extract was investigated to assess the quality control of the natural antibacterial resource, and quantitative estimation of several major phenolics in the extract with the established HPLC condition was also performed. The results showed individual contents of 0.08%–0.57% w/w of EtOAc extract for the major phenolics in the materials examined.

**Keywords:** licorice; *Glycyrrhiza uralensis*; flavonoid; 2-aryl-3-methylbenzofuran; VRE; antibacterial effect; HPLC

---

## 1. Introduction

Infectious diseases caused by multidrug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) are serious problems worldwide [1]. Although *Enterococcus* bacteria are considered ordinary components in the healthy human intestinal flora, they are responsible for complicated urinary tract infections and serious endocarditis [2]. *Enterococcus faecium* and *Enterococcus faecalis* account for >95% of *Enterococcus* isolates from clinical cultures [3], and only a few drugs such as linezolid and a combination of quinupristin and dalbapristin are used clinically for VRE [4]. Since the adverse effects of these drugs have been revealed and drug resistance to them may appear soon, the development of a new group of low toxicity antibacterial agents is needed. Licorice has been used as a food sweetener and is one of the oldest and most frequently used crude drugs in traditional medicine, particularly in Asian countries. A variety of pharmaceutical functions, such as antiulcer, anti-inflammatory, antiviral, and anticarcinogenic activities have been reported for licorice constituents [5–8], and the antibacterial effects of licorice phenolics have been demonstrated for various bacterial species [9–14]. The effect of a compound isolated from licorice, gancaonin I (**1**), on VRE was also demonstrated in a previous study [15].

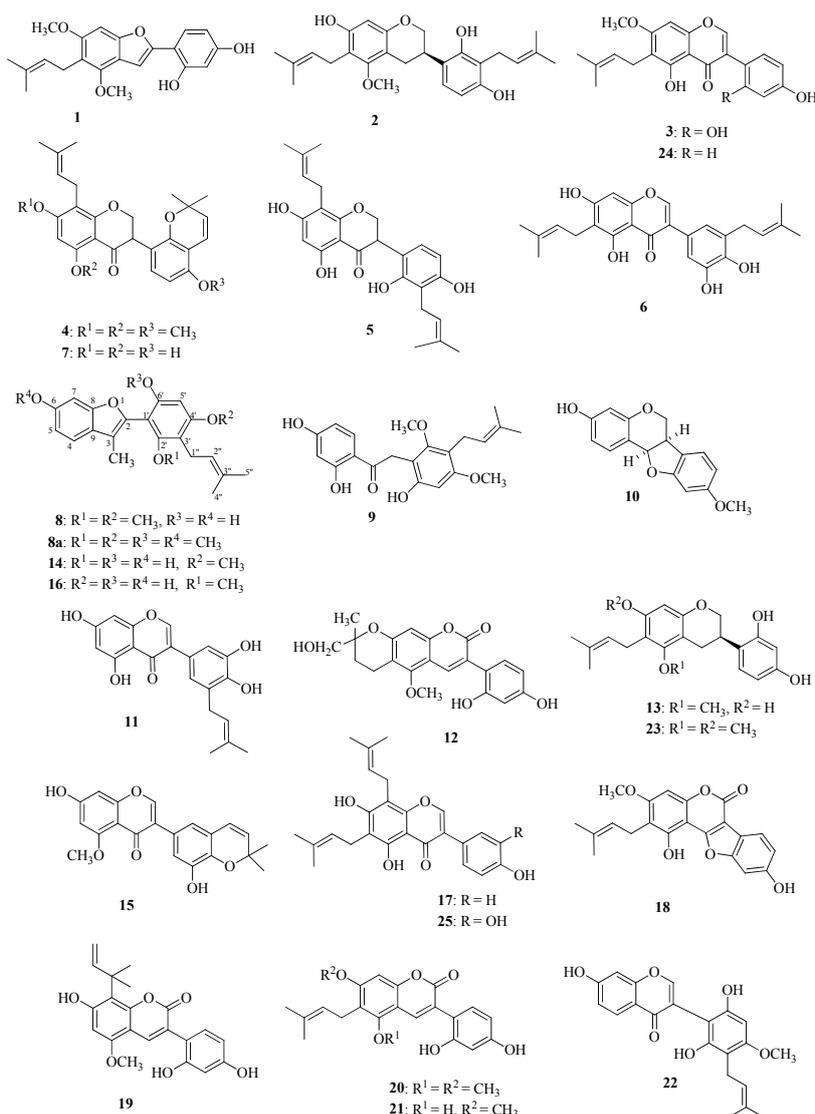
Our continuous studies have revealed the antibacterial effects of several licorice phenolics on MRSA, particularly those with both  $\gamma,\gamma$ -dimethylallyl (prenyl) and hydroxyl groups [16]. Licoricidin (**2**) has the same structural features and displays a suppressive effect on oxacillin resistance shown by MRSA [16]. We also reported the anti-VRE effects of several licorice phenolics in a previous study [17]. Our further investigations have led to the isolation of 16 phenolic compounds including two new compounds with rarely occurring 2-aryl-3-methylbenzofuran structures. This paper explains the structural determination of the new compounds and the effects of those phenolics on two VRE stains. In addition, an analytical condition for high-performance liquid chromatography (HPLC) to simultaneously analyze polyphenolic constituents in the EtOAc extract was established for quality control of the antibacterial resource, and several major phenolics in the extract were quantitated using the established HPLC condition.

## 2. Results and Discussion

A part of the EtOAc extract obtained from powdered licorice was subjected to column chromatography on ODS-gel and eluted with increasing concentrations of MeOH in H<sub>2</sub>O and then with

increasing concentrations of  $\text{CHCl}_3$  in MeOH. The eluate with 50%  $\text{CHCl}_3$  in MeOH was subjected to column chromatography on MCI-gel CHP-20P with increasing concentrations of MeOH in  $\text{H}_2\text{O}$ . Fractions from the column were purified by preparative HPLC, to give 16 licorice phenolics, including licoricidin (**2**) [18], 7-*O*-methylluteone (**3**) [19], glyasperin J trimethyl ether (**4**) [20], 3'-( $\gamma,\gamma$ -dimethylallyl)-kievitone (**5**) [21], isoangustone A (**6**) [22], glyasperin J (**7**) [20], compound A (**8**), licoriphenone (**9**) [23], demethylhomopterocarpan (**10**) [24], glycyrrhisofavone (**11**) [25], licopyranocoumarin (**12**) [26], glyasperin C (**13**) [27], compound B (**14**), glycyrrhiza-isoflavone B (**15**) [28], glycybenzofuran (**16**) [29], and 8-( $\gamma,\gamma$ -dimethylallyl)-wightone (**17**) [30] from the respective fractions. The structures of **2–7**, **9–13**, and **15–17** (Figure 1) were identified by comparisons of spectral data with values reported in the literature [18–30], whereas the two arylbenzofurans, temporarily named compounds A (**8**) and B (**14**) (Figure 1), are new, and their structure elucidations are described below.

**Figure 1.** Structures of the compounds **1–25** found in the EtOAc extract of *Glycyrrhiza uralensis* roots and stolons (Tohoku licorice). Structures **2–17** are the compounds isolated in this study <sup>a</sup>.



<sup>a</sup> The configuration at C-3 in each of compounds **4**, **5**, and **7** was not determined in the previous papers [20,21].

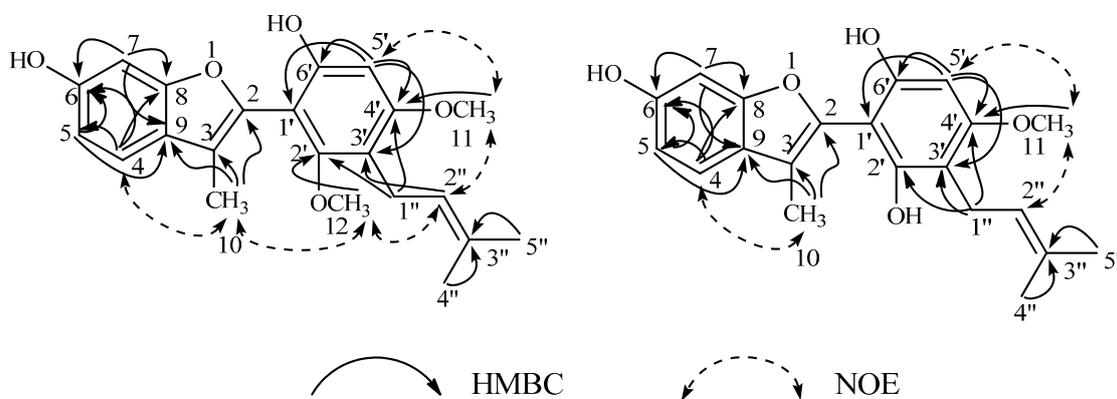
### 2.1. Structures of the New Compounds

Compound A (**8**): This compound was obtained as a light brown powder. Its molecular formula was  $C_{22}H_{24}O_5$ , based on the  $[M + H]^+$  ion peak in the high-resolution fast-atom bombardment mass spectrometry (HR-FAB-MS). The ultraviolet (UV) spectrum of **8** showed absorption maxima at 214 (log  $\epsilon$  4.11), 238 (4.01), and 305 nm (4.52), indicating structural similarity to those of the known compounds glycybenzofuran (**16**) and licocoumarone (**18**) with 2-arylbenzofuran skeletons. The  $^1H$  nuclear magnetic resonance (NMR) spectrum of **8** (in acetone- $d_6$ ) showed resonances of three aromatic protons at  $\delta_H$  7.30 (d,  $J = 8.4$  Hz, H-4), 6.87 (d,  $J = 2.4$  Hz, H-7), and 6.77 (dd,  $J = 2.4, 8.4$  Hz, H-5), forming an ABX spin system, and a one-proton singlet at  $\delta_H$  6.41 (H-5'). The spectrum also showed four sets of proton resonances at  $\delta_H$  5.16 (1H, t,  $J = 6.6$  Hz, H-2''), 3.32 (2H, d,  $J = 6.6$  Hz, H-1''), 1.60 (3H, s), and 1.70 (3H, s) ( $2 \times CH_3$  at C-3''), which are assignable to those of a  $\gamma,\gamma$ -dimethylallyl (prenyl) group. In addition, proton resonances characteristic of two methoxyl groups at  $\delta_H$  3.82 and 3.35 (3H each, s) and one methyl group at  $\delta_H$  1.89 (3H, s) were seen in the aliphatic region of the spectrum.

The  $^{13}C$ -NMR spectrum showed six carbon resonances due to oxygenated  $sp^2$  carbons ( $\delta_C$  160.0, 158.9, 156.2, 155.8, 153.1, and 145.7) and eight carbon resonances attributable to non-oxygenated  $sp^2$  carbons [ $\delta_C$  123.0, 119.6, 114.2 (2C) 111.5, 102.1 97.9, and 96.6]. The spectrum also showed five carbon resonances due to the prenyl group ( $\delta_C$  17.2, 22.5, 25.3, 124.3, and 129.9) and two methoxyl groups ( $\delta_C$  60.7 and 55.0). In addition to these resonances, the spectrum showed a methyl carbon resonance ( $\delta_C$  7.9), ascribable to the methyl group at C-3 of the 2-arylbenzofuran structure.

The assignments of these proton and carbon resonances were substantiated by the heteronuclear single quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) spectral data as summarized in Table 1. Key HMBC correlations among them and the nuclear Overhauser effect spectroscopy (NOESY) correlations indicating the locations of the respective substituents on the 2-arylbenzofuran skeleton are shown in Figure 2.

**Figure 2.** HMBC and NOESY correlations observed for compounds **8** and **14**.



**Table 1.**  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR assignments, HSQC and HMBC correlations for compounds (8) and (14) (600 MHz for  $^1\text{H}$  and 151 MHz for  $^{13}\text{C}$ , acetone- $d_6$ , 27 °C) <sup>a,b</sup>.

Position	4'-O-Methylglycybenzofuran (8)				Neoglycybenzofuran (14)			
	$\delta_{\text{C}}$	HSQC <sup>a</sup>	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	HMBC <sup>b</sup>	$\delta_{\text{C}}$	HSQC <sup>a</sup>	$\delta_{\text{H}}$ ( <i>J</i> in Hz)	HMBC <sup>b</sup>
C-2	145.7	C		H-10	145.5	C		H-10
C-3	114.2	C		H-10	114.4	C		H-10
C-4	119.6	CH	7.30, d (8.4)		119.3	CH	7.23, d (9.0)	
C-5	111.5	CH	6.77, dd (2.4, 8.4)	H-4	111.4	CH	6.71, dd (2.4, 9.0)	
C-6	155.8	C		H-5, 7	155.7	C		H-4, 5, 7
C-7	97.9	CH	6.87, d (2.4)		97.8	CH	6.82, d (2.4)	
C-8	156.2	C		H-4, 7	156.2	CH		H-4, 7
C-9	123.0	C		H-5, 7, 10	123.8	CH		H-5, 7, 10
C-10	7.9	CH <sub>3</sub>	1.89 s		8.7	CH <sub>3</sub>	1.97 s	
C-1'	102.1	C		H-5'	103.5	C		H-5'
C-2'	158.9	C		H-1''	159.1	C		H-1''
C-3'	114.2	C		H-5', H-2''	112.9	C		H-5', 1'', 2''
C-4'	160.0	C		H-5', H-1''	160.0	C		H-5', 1''
C-5'	96.6	CH	6.41 s		98.7	CH	6.28 s	
C-6'	153.1	C		H-5'	155.7	C		H-5'
C-1''	22.5	CH <sub>2</sub>	3.32, d (6.6)		22.9	CH <sub>2</sub>	3.13, d (6.6)	
C-2''	124.3	CH	5.16, t (6.6)		124.6	CH	5.12, t (6.6)	
C-3''	129.9	C		H-4'', H-5''	129.7	C		H-4'', 5''
C-4''	17.2	CH <sub>3</sub>	1.60 s		17.8	CH <sub>3</sub>	1.69 s	
C-5''	25.3	CH <sub>3</sub>	1.70 s		25.5	CH <sub>3</sub>	1.63 s	
-OCH <sub>3</sub>	60.7	CH <sub>3</sub>	3.82 s	H-11	60.6	CH <sub>3</sub>	3.28 s	H-11
-OCH <sub>3</sub>	55.0	CH <sub>3</sub>	3.35 s	H-12				

<sup>a</sup> HSQC, shows the relationship between a proton directly connected to a carbon. <sup>b</sup> HMBC correlations, optimized for 5 Hz, are from proton(s) stated to the optimized carbons.

The locations of the hydroxyl and methoxyl groups, and the prenyl group on the B-ring of this compound were shown by the following HMBC correlations. Correlation of the methylene proton resonance at  $\delta_{\text{H}}$  3.32 (H-1'') of the prenyl moiety and an -OCH<sub>3</sub> resonance ( $\delta_{\text{H}}$  3.82) with a common oxygenated aromatic carbon resonance at  $\delta_{\text{C}}$  160.0 (C-4') and also the correlations of the same methylene proton resonance (H-1'') and a methoxyl proton resonance ( $\delta_{\text{H}}$  3.35) with a common oxygenated carbon resonance at  $\delta_{\text{C}}$  158.9 (C-2'). The carbon resonance at  $\delta_{\text{C}}$  160.0 (C-4') was also correlated with the singlet proton resonance at  $\delta_{\text{H}}$  6.41 (H-5', directly correlated with the C-5 carbon resonance at  $\delta_{\text{C}}$  96.6 in the HSQC spectrum), and this proton resonance was also correlated with an oxygenated aromatic carbon at  $\delta_{\text{C}}$  153.1 (C-6'), which was not correlated with any methoxyl proton resonance. The H-5' resonance was also correlated with the carbon resonances at  $\delta_{\text{C}}$  114.2 (C-3') and  $\delta_{\text{C}}$  102.1 (C-1') of the same aromatic ring. The last two carbon resonances were discriminated by a correlation of the methylene proton resonance (H-1'') to the carbon resonance at  $\delta_{\text{C}}$  114.2 (C-3'). The

sequence C-1' ( $\delta_C$  102.1)–C-2' ( $\delta_C$  158.9, with a methoxyl group)–C-3' ( $\delta_C$  114.2, with the prenyl group)–C-4' ( $\delta_C$  160.0, with a methoxyl group)–C-5' ( $\delta_C$  96.6)–C-6' ( $\delta_C$  153.1) was thus assigned for the B-ring. In addition, the NOESY spectrum of this compound showed correlations of the methine proton resonance at  $\delta_H$  5.16 (H-2" of the prenyl moiety) with the two methoxyl resonances at  $\delta_H$  3.82 (-OCH<sub>3</sub> at C-4') and  $\delta_H$  3.35 (-OCH<sub>3</sub> at C-2'), and the former methoxyl resonance also showed a correlation with the proton resonance at  $\delta_H$  6.41 (C-5'), in agreement with the sequence described above.

The presence of a methyl group at C-3 was clearly indicated by the HMBC correlations of the methyl proton resonance at  $\delta_H$  1.89 (H-10) with the carbon resonance at  $\delta_C$  145.7 (C-2), 114.2 (C-3), and 123.0 (C-9). The NOESY correlations of this proton resonance with the methoxyl proton resonance at  $\delta_H$  3.35 (-OCH<sub>3</sub> at C-2'), and with the aromatic proton resonance at  $\delta_H$  7.30 (H-4), are also consistent with the location C-3 of the methyl group. The coupling patterns of H-4 [ $\delta_H$  7.30 (d,  $J$  = 8.4 Hz)], H-5 [ $\delta_H$  6.77 (dd,  $J$  = 2.4, 8.4 Hz)], and H-7 [ $\delta_H$  6.87 (d,  $J$  = 2.4 Hz)] resonances, forming an ABX system, indicated the location C-6 for the hydroxyl group. These data, and also the remaining HMBC correlations, satisfied the 2-aryl-3-methyl-6-hydroxybenzofuran structure.

Because structure **8** assigned to compound A was an analog of a compound reported previously, glycybenzofuran (**16**), the corresponding methylated products of compounds **8** and **16** were compared. As a result, product **8a** from **8** was the same as that obtained by methylation of **16**, as expected. The structure of compound A was thus substantiated to be 4'-O-methylglycybenzofuran (**8**).

Compound B (**14**): Compound B was obtained as a light brown powder. The molecular formula C<sub>21</sub>H<sub>22</sub>O<sub>5</sub>, which was the same as that of glycybenzofuran (**16**), was detected by HR-FAB-MS. The UV spectrum of **14** (in MeOH) showed absorption maxima at 210 (log  $\epsilon$  4.09), 238 (4.21), and 300 nm (4.30), where the spectral feature characteristic of the 2-arylbenzofuran skeleton was seen in the spectra of compounds **8** and **16**. The aromatic region of the <sup>1</sup>H-NMR spectrum of **14** (in acetone-*d*<sub>6</sub>) showed a one-proton singlet at  $\delta_H$  6.28 and the three proton resonances forming an ABX spin system at  $\delta_H$  7.23 (d,  $J$  = 8.4 Hz, H-4), 6.82 (d,  $J$  = 2.4 Hz, H-7), and 6.71 (dd,  $J$  = 2.4, 8.4 Hz, H-5). The spectrum also exhibited characteristic resonances of a prenyl moiety at  $\delta_H$  3.13 (2H, d,  $J$  = 6.6 Hz, H-1"), 5.12 (1H, t,  $J$  = 6.6 Hz, H-2"), 1.69 (3H, s), and 1.63 (3H, s) (*gem*-dimethyl at C-3"). In addition, the spectrum exhibited a methyl proton resonance at  $\delta_H$  1.97 (3H, s) and a methoxyl resonance at  $\delta_H$  3.28 (3H, s). These data indicate structural similarity of **14** to that of **8** except for the number of the methoxyl resonances. That is, **14** had a structure isomeric to **16**, concerning the placement of the methoxyl group.

The <sup>13</sup>C-NMR spectrum of **14** showed resonances of 14 *sp*<sup>2</sup> carbons attributable to the 2-arylbenzofuran skeleton composed of six oxygenated carbons [ $\delta_C$  160.0, 159.1, 156.2, 155.7 (2C), and 145.5] and eight non-oxygenated carbons ( $\delta_C$  123.8, 119.3, 114.4, 112.9, 111.4, 103.5, 98.7, and 97.8). The spectrum also showed a methyl carbon resonance at  $\delta_C$  8.7, a methoxyl carbon resonance at  $\delta_C$  60.6, and five carbon resonances due to a prenyl unit ( $\delta_C$  17.8, 22.9, 25.5, 124.6, and 129.7).

The HMBC spectrum (Table 1 and Figure 2) showed correlations  $\delta_C$  159.1 (C-2')– $\delta_H$  3.13 (H-1" of prenyl at C-3')– $\delta_C$  160.0 (C-4')– $\delta_H$  3.28 (OCH<sub>3</sub> at C-4'), and also the correlations  $\delta_C$  160.0 (C-4')– $\delta_H$  6.28 (H-5')– $\delta_C$  155.7 (C-6'), and  $\delta_H$  6.28 (H-5')– $\delta_C$  103.5 (C-1'). Furthermore, the NOESY spectrum showed correlations  $\delta_H$  5.12 (H-2" of prenyl at C-3')– $\delta_H$  3.28 (-OCH<sub>3</sub> at C-4')– $\delta_H$  6.28 (H-5') (Figure 2). These correlations clearly indicate the sequence C-1'–C-6' (with -OH)–C-5'–C-4' (with -OCH<sub>3</sub>)–C-3' (with prenyl)–C-2' (with -OH) of the B-ring structure.

The presence of the methyl group at C-3 was indicated by the HMBC correlations from the methyl proton resonance (H-10) at  $\delta_{\text{H}}$  1.97 with C-2 ( $\delta_{\text{C}}$  145.5), C-3 ( $\delta_{\text{C}}$  114.4), and C-9 ( $\delta_{\text{C}}$  123.8) and the NOESY correlations between the methyl resonance at  $\delta_{\text{H}}$  1.97 and H-4 at  $\delta_{\text{H}}$  7.23. The resonances of H-4, H-5, and H-7, forming an ABX system as shown by the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum, indicated the location of a hydroxyl group at C-6, and the HMBC correlations (Figure 2) concerning these aromatic proton resonances also satisfied the location C-6 of the hydroxyl group. Based on these findings, structure **14**, which was isomeric to **16**, was assigned to compound B which accordingly was named neoglycybenzofuran. Methylation of **14** afforded **8a** and thus substantiated the structure **14** for neoglycybenzofuran.

## 2.2. Antibacterial Effects of Licorice Phenolics on VRE

The antibacterial effects of the licorice phenolics on the two species of VRE, *E. faecium* FN-1 and *E. faecalis* NCTC 12201, were estimated using the liquid dilution method as described previously [17]. The results summarized in Table 2 reveal that almost all of the licorice phenolics examined showed antibacterial effects [minimum inhibitory concentration (MIC),  $1.9 \times 10^{-5}$ – $3.5 \times 10^{-4}$  M] on the VRE strains, and several ones among them showed noticeable anti-VRE effects (Table 2).

**Table 2.** Antibacterial effects of licorice phenolics on *Enterococci* (estimated minimum inhibitory concentrations, MIC) <sup>a</sup>.

Compounds	Number of -OH Groups	Number of Prenyl Groups	MIC ( $10^{-5}$ M)	
			<i>Enterococcus faecium</i> FN-1	<i>Enterococcus faecalis</i> NCTC12201
<b>Isoflavones</b>				
7- <i>O</i> -Methyluteone ( <b>3</b> )	3	1	8.7	8.7
Isoangustone A ( <b>6</b> )	4	2	3.8	3.8
Glycyrrhisofavone ( <b>11</b> )	4	1	9.0	9.0
Glycyrrhiza-isoflavone B ( <b>15</b> )	2	0	35	35
8-( $\gamma,\gamma$ -Dimethylallyl)-wightone ( <b>17</b> )	3	2	1.9	3.8
Glicoricone ( <b>22</b> ) <sup>a</sup>	3	1	>35	>35
6,8-Diprenylorobol ( <b>25</b> ) <sup>a</sup>	4	2	30	30
<b>Isoflavans</b>				
Licoricidin ( <b>2</b> )	3	2	1.9	1.9
Glyasperin C ( <b>13</b> )	3	1	4.5	4.5
<b>Isoflavanones</b>				
Glyasperin J trimethyl ether ( <b>4</b> )	0	1	14	14
3'-( $\gamma,\gamma$ -Dimethylallyl)-kiewitone ( <b>5</b> )	4	2	3.8	3.8
Glyasperin J ( <b>7</b> )	3	1	7.5	7.5
<b>3-Arylcoumarins</b>				
Licopyranocoumarin ( <b>12</b> )	2	0	>33	33
Glycyrin ( <b>20</b> ) <sup>a</sup>	2	1	4.2	8.4
Glycy coumarin ( <b>21</b> ) <sup>a</sup>	3	1	4.3	4.3
<b>Coumestans</b>				
Glycyrol ( <b>18</b> ) <sup>a</sup>	2	1	35	>35
<b>Pterocarpan</b>				
Demethylhomopterocarpan ( <b>10</b> )	1	0	12	12

Table 2. Cont.

Compounds	Number of -OH Groups	Number of Prenyl Groups	MIC ( $10^{-5}$ M)	
			<i>Enterococcus faecium</i> FN-1	<i>Enterococcus faecalis</i> NCTC12201
<b>2-Aryl-3-methylbenzofurans</b>				
Gancaonin I (1) <sup>a</sup>	2	1	4.5	4.5
4'-O-Methylglycybenfuran (8)	2	1	8.7	8.7
Noeglycybenzofuran (14)	3	1	4.5	4.5
Glycybenzofuran (16)	3	1	18	18
<b>Benzylphenylketones</b>				
Licoriphenone (9)	3	1	>34	34
<b>Standard antibacterial agents</b>				
Vancomycin <sup>a</sup>			>6.9	>6.9
Linezolid <sup>a</sup>			0.74	0.74
<b>EtOAc extract from Tohoku licorice</b>			16 $\mu\text{g/mL}$	32 $\mu\text{g/mL}$

<sup>a</sup> Data taken from [17].

Among these compounds, licoricidin (2) (isoflavan) showed the most potent effects against both *E. faecalis* and *E. faecium* (MIC,  $1.9 \times 10^{-5}$  M). 8-( $\gamma,\gamma$ -Dimethylallyl)-wighteone (17) (isoflavone), isoangustone A (6) (isoflavone), 3'-( $\gamma,\gamma$ -dimethylallyl)-kieveitone (5) (isoflavanone), glyasperin C (13) (isoflavan), and neoglycybenzofuran (14) (new, 2-aryl-3-methylbenzofuran) also showed anti-VRE effects with MICs of  $1.9 \times 10^{-5}$ – $4.5 \times 10^{-5}$  M, respectively. All of these compounds have three or more phenolic hydroxyl groups and at least one prenyl group. In contrast, several other compounds such as licoriphenone (9) showed relatively weaker effects, and they had analogous structural features. Further experiments are required to clarify the structural factors responsible to the antibacterial effects.

The antibacterial effects of the EtOAc extract were comparable to those of potent anti-VRE constituents. Potential synergy and/or additive effects between the purified phenolics remain to be determined.

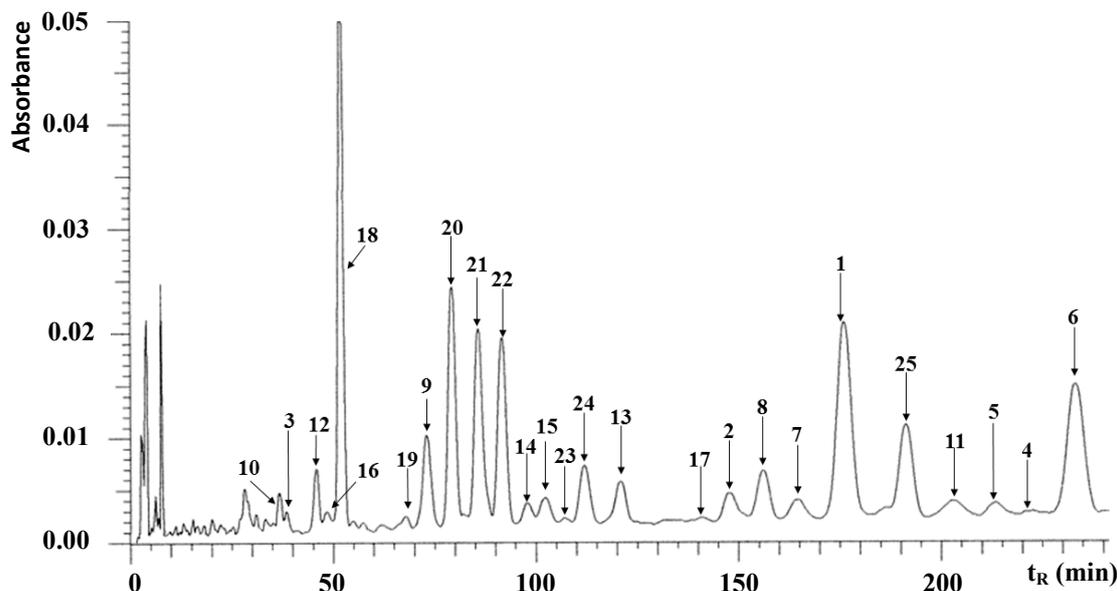
### 2.3. HPLC Analyses of Anti-VRE Phenolics for the Evaluation of EtOAc Extract from *G. uralensis* as a Source of Antibacterial Agent

Because of the important uses of licorice in traditional medicine, qualitative and quantitative analyses of licorice constituents and licorice products have been reported [31–36]. Remarkable anti-VRE effects of several licorice phenolics shown in our current and previous studies [17] suggested requirements of the identification and quantitation of those constituents. We therefore developed an HPLC-UV method for the simultaneous detection of major isolated phenolic constituents in EtOAc extract from *G. uralensis*, in order to evaluate the quality of the extract as a source of antibacterial agent.

The HPLC-UV profile of the EtOAc extract from Tohoku licorice used in the present study under the established condition is shown in Figure 3. Each constituent in the HPLC profile was identified by comparisons of its retention time, UV and MS spectra (data not shown) with those of the isolated one. The elution order of the identified constituents was as follows: demethylhomopterocarpan (10,  $t_R$  38.6 min), 7-O-methyllyuteone (3,  $t_R$  41.2 min), licopyranocoumarin (12,  $t_R$  46.0 min), glycybenzofuran (16,  $t_R$  46.6 min), glycyrol (18,  $t_R$  52.9 min), licoaryl coumarin (19,  $t_R$  68.0 min), licoriphenone (9,

$t_R$  73.2 min), glycyrin (**20**,  $t_R$  79.4 min), glycycomarin (**21**,  $t_R$  85.9 min), glicoricone (**22**,  $t_R$  91.8 min), neoglycybenzofuran (**14**,  $t_R$  98.1 min), glycyrrhiza-isofavone B (**15**,  $t_R$  102.4 min), glyasperin D (**23**,  $t_R$  107.4 min), gancaonin G (**24**,  $t_R$  112.1 min), glyasperin C (**13**,  $t_R$  121.0 min), 8-( $\gamma,\gamma$ -dimethylallyl)-wighteone (**17**,  $t_R$  141.1 min), licoricidin (**2**,  $t_R$  147.9 min), 4'-*O*-methylglycybenzofuran (**8**,  $t_R$  156.1 min), glyasperin J (**7**,  $t_R$  164.5 min), gancaonin I (**1**,  $t_R$  176.1 min), 6,8-diprenylorobol (**25**,  $t_R$  191.3 min), glycyrrhisofavone (**11**,  $t_R$  202.9 min), 3'-( $\gamma,\gamma$ -dimethylallyl)-kievitone (**5**,  $t_R$  213.5 min), glyasperin J trimethyl ether (**4**,  $t_R$  222.8 min), and isoangustone A (**6**,  $t_R$  233.2 min).

**Figure 3.** HPLC-UV chromatogram of *G. uralensis* (Tohoku licorice) EtOAc extract at 280 nm <sup>a-c</sup>.



<sup>a</sup> Column, YMC-Pack Pro C18 (6.0 mm i.d.  $\times$  150); mobile phase, H<sub>2</sub>O/MeCN/MeCOOH (55:40:5, v/v/v); flow rate, 1.0 mL/min; oven temperature, 40 °C; detector, Hitachi L2455. <sup>b</sup> The numbers represent the isolated compounds, as displayed in Figure 1. <sup>c</sup> The configuration of each of the optically active constituents was not reflected in the present analytical conditions.

Quantitative analysis of several compounds was performed under the same HPLC condition, and the amounts of the major phenolic constituents are shown in Table 3. Among these major phenolics, gancaonin I (**1**) and isoangustone A (**6**) showed potent anti-VRE effects.

**Table 3.** Contents of major licorice phenolics in *G. uralensis* (Tohoku licorice) EtOAc extract.

Compound	Content (% w/w) <sup>a</sup>
Glycyrol ( <b>18</b> )	0.54 $\pm$ 0.036
Gancaonin I ( <b>1</b> )	0.49 $\pm$ 0.025
Isoangustone A ( <b>6</b> )	0.34 $\pm$ 0.031
Glycyrin ( <b>20</b> )	0.26 $\pm$ 0.015
Glycycomarin ( <b>21</b> )	0.24 $\pm$ 0.010
Glicoricone ( <b>22</b> )	0.18 $\pm$ 0.023
6,8-Diprenylorobol ( <b>25</b> )	0.094 $\pm$ 0.013
Licoriphenone ( <b>9</b> )	0.082 $\pm$ 0.017

<sup>a</sup> The value was given as the mean  $\pm$  standard deviation (SD) based on the triplicate experiments.

### 3. Experimental Section

#### 3.1. General Information

UV spectra were recorded on a V-530 spectrometer (JASCO, Tokyo, Japan). Measurements of electrospray ionization mass spectra were taken on an API-4000 instrument (AB Sciex, Framingham, MA, USA) and high-resolution fast atom bombardment-mass spectroscopy (HR-FAB-MS) was conducted on a JMS-700 MStation (JEOL, Tokyo, Japan) with a mixture of *m*-nitrobenzyl alcohol and dithiothreitol as the matrix.  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectra were recorded on an INOVA 600AS instrument (600 MHz for  $^1\text{H}$  and 151 MHz for  $^{13}\text{C}$ ; Agilent, Santa Clara, CA, USA). Chemical shifts of the resonances in these spectra were adjusted using those of the solvent resonances [ $\delta_{\text{H}}$  2.04 and  $\delta_{\text{C}}$  29.8 for  $(\text{CD}_3)_2\text{CO}$ ] and are given in  $\delta$  (ppm) values. Analytical HPLC-DAD to monitor purification of the constituents was conducted on an ODS-A 302 (4.6 mm i.d.  $\times$  250 mm; YMC, Kyoto, Japan) column at 40 °C in an oven with 10 mM  $\text{H}_3\text{PO}_4$ /10 mM  $\text{KH}_2\text{PO}_4$ /MeCN (35:35:30, v/v/v, isocratic mode) as the eluent. A Hitachi L-2455 detector was used for monitoring UV absorption at 280 nm, and the flow rate was set at 1.0 mL/min. Preparative HPLC was performed on an ODS-A324 (10 mm i.d.  $\times$  300 mm; YMC) column at 40 °C in an oven with  $\text{H}_2\text{O}$ /MeCN/MeCOOH (45:50:5, v/v/v) as the eluent. UV absorption at 280 nm was used for HPLC detection, and the flow rate was set at 2.0 mL/min. The procedure for the simultaneous HPLC analyses of the phenolic constituents in the EtOAc extract is described separately (see below). Silica gel (YMC), Toyopearl HW-40 (coarse grade; TOSOH, Tokyo, Japan), YMC-gel ODS-A (S, 75  $\mu\text{m}$ ; YMC), and MCI-gel CHP-20P (Mitsubishi Chemical, Tokyo, Japan) were used for column chromatography.

#### 3.2. Plant Material

The crude drug used in this study was Tohoku licorice, which is the dried roots and stolons of *Glycyrrhiza uralensis* Fisch. ex DC, purchased from Tochimoto-tenkai-do (Osaka, Japan) (lot no. 002009037), and the GU-07112011(NEL) specimen was kept at the Medicinal Plant Garden, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

#### 3.3. Extraction and Isolation

Licorice (1.0 kg) was pulverized and dipped in *n*-hexane (3 L  $\times$  2). Then, the defatted material was treated with EtOAc (3 L  $\times$  2) to give the extract (46.4 g). Part (40 g) of the EtOAc extract was subjected to column chromatography on ODS-gel (2.2 i.d.  $\times$  75 cm) with increasing concentrations of MeOH in  $\text{H}_2\text{O}$  and then with increasing concentrations of  $\text{CHCl}_3$  in MeOH. The eluate with 50%  $\text{CHCl}_3$  in MeOH (3.6 g) was subjected to column chromatography on MCI-gel CHP-20P (2.2 i.d.  $\times$  45 cm) with increasing concentrations of MeOH in  $\text{H}_2\text{O}$ . Fractions 94 (42 mg), 96 (40 mg), 161 (38 mg), 230 (31 mg), 231 (30 mg), 234 (28 mg), 327 (24 mg), and 337 (22 mg) were respectively purified by preparative HPLC on YMC-Pack A-324 (10 mm i.d.  $\times$  300 mm; 2.5 mL/min;  $\text{H}_2\text{O}$ /MeCN/MeCOOH, 55:40:5, v/v/v; isocratic mode; monitored at 280 nm) to give the following phenolics: licoricidin (**2**, 3.1 mg), 7-*O*-methylluteone (**3**, 2 mg), and glyasperin J trimethyl ether (**4**, 1.5 mg) from fraction 94; 3'-( $\gamma,\gamma$ -dimethylallyl)-kieveitone (**5**, 4.0 mg), isoangustone A (**6**, 9.0 mg), glyasperin J (**7**, 2.3 mg),

compound A (**8**, 3.2 mg), and licoriphenone (**9**, 1.9 mg) from fraction 96; demethylhomopterocarpan (**10**, 1.2 mg) from fraction 161; glycyrrhisofavone (**11**, 5.0 mg) from fraction 230; licopyranocoumarin (**12**, 4.9 mg), glyasperin C (**13**, 3.0 mg), and compound B (**14**, 2.0 mg) from fraction 231; glycyrrhiza-isofavone B (**15**, 1.6 mg) from fraction 234; glycybenzofuran (**16**, 1.8 mg) from fraction 327; and 8-( $\gamma,\gamma$ -dimethylallyl)-wighteone (**17**, 1.5 mg) from fraction 337. The purity of each of the isolated compounds were >98%, as estimated by HPLC and  $^1\text{H-NMR}$ .

### 3.4. Spectral Data

Compound A (4'-*O*-Methylglycybenzofuran, **8**): This compound was obtained as a light brown powder;  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  (see Table 1); HR-FAB-MS  $m/z$  369.1702 ( $[\text{M} + \text{H}]^+$ ), (Calculated for  $\text{C}_{22}\text{H}_{24}\text{O}_5$ , 369.1709).

Compound B (Neoglycybenzofuran, **14**): This compound was obtained as a light brown powder;  $^1\text{H-}$  and  $^{13}\text{C-NMR}$  (see Table 1); HR-FAB-MS  $m/z$  355.1546 ( $[\text{M} + \text{H}]^+$ ), (Calculated for  $\text{C}_{21}\text{H}_{22}\text{O}_5$ , 355.1549).

### 3.5. Methylation of Compounds A and B, and Glycybenzofuran

Trimethylsilyldiazomethane solution (1 mL) was added to a solution of **8** (1 mg) in EtOH (0.1 mL), and the mixture was kept for 3 h at room temperature. After evaporating the solvent, the remaining product was purified by TLC on silica gel (Merck, silica gel F254) ( $\text{CHCl}_3$ -MeOH, 15:1, v/v) to give a methyl derivative of compound A (**8a**). Detection was effected by UV absorption at 254 nm.  $^1\text{H-NMR}$  (600 MHz, acetone- $d_6$ ):  $\delta_{\text{H}}$  1.99, 1.74, 1.65 (each 3H, s,  $-\text{CH}_3 \times 3$ ), 3.27 (2H, d,  $J = 6.6$  Hz, H-1"), 3.38, 3.82 (each 3H, s,  $-\text{OCH}_3 \times 2$ ), 5.17 (1H, t, H-2"), 6.40 (1H, s, H-5'), 6.80 (1H, dd,  $J = 2.4, 8.4$  Hz, H-5), 6.88 (1H, d,  $J = 2.4$  Hz, H-7), 7.35 (1H, d,  $J = 8.4$  Hz, H-4). The identical compound was obtained by treating compound B (**14**) and glycybenzofuran (**16**) with trimethylsilyldiazomethane in analogous ways.

### 3.6. Antibacterial Assay

Estimations of the antibacterial effects of licorice phenolics on the VRE *E. faecium* FN-1 and *E. faecalis* NCTC 12201 used in this study were conducted using VRE kindly provided by Y. Ike, Gunma University. The bacterial cells were precultured in Mueller-Hinton broth at 37 °C under aerobic conditions. They were incubated in the presence of compounds with the concentrations obtained by serial two-fold dilution at 37 °C without shaking in the same broth for 24 h on microplates as shown in a previous paper [17], and their MICs were estimated as the lowest concentrations where the bacterial cells were not observed visually as reported previously [16,17], and were given based on triplicate experiments. DMSO was used for dissolving compounds hardly soluble in water, and the final concentrations were set at <1%, where DMSO has no effect. The positive control, linezolid, was dissolved in water.

### 3.7. Simultaneous HPLC Analysis of Phenolic Constituents in the EtOAc Extract of Licorice

Simultaneous analysis of licorice phenolics was carried out on an HPLC-DAD D-2000 HSM system, composed of an L-2130 pump (Hitachi, Tokyo, Japan) and an L-2455 DAD (Hitachi). The DAD was set for obtaining UV spectral data from 200 to 400 nm, and chromatograms at 280 nm were used for the quantitative analyses. The column used was an YMC-Pack pro C18 (6.0 mm i.d. × 150 mm) and was set in an oven at 40 °C. The mobile phase consisted of H<sub>2</sub>O/MeCN/MeCOOH (55:40:5, v/v/v), and the flow rate was set at 1.0 mL/min. Quantitation of **1**, **6**, **9**, **18**, **20**, **21**, **22**, and **25** was based on the HPLC profile monitored at 280 nm.

Licorice (10 g) was pulverized and extracted with EtOAc (100 mL × 3). Approximately 10 mg of the dried extract powder was dissolved in 10 mL of MeOH and filtered with a 0.45 µm PTFE membrane filter prior to injection (8 µL of the filtrate at 1 mg/mL) was applied to HPLC analysis. Stock solutions of eight licorice phenolics (**1**, **6**, **9**, **18**, **20**, **21**, **22** and **25**) were prepared at 0.1 mg/mL in MeOH, and diluted in series (from 0.1 to 0.001 mg/mL) to produce eight individual standard curves, for which the correlation coefficients were determined between 0.991 and 0.999 under the described HPLC conditions.

## 4. Conclusions

Our present investigation on the EtOAc extract of *G. uralensis* led to the purification of 16 compounds. Among the compounds obtained, two new compounds, **8** and **14**, had 2-aryl-3-methylbenzofuran structures, which rarely occur in Nature. The isolated phenolics were categorized into isoflavones (**3**, **6**, **11**, **15**, and **17**), isoflavans (**2** and **13**), isoflavanones (**4**, **5**, and **7**), a 3-arylcoumarin (**12**), a pterocarpan (**10**), 2-aryl-3-methylbenzofurans (**8**, **14**, and **16**), and a benzylphenylketone (**9**). As shown in our previous studies, licorice phenolics possess remarkable antibacterial effects against MRSA [16] and VRE [17]. The effects of the licorice phenolics isolated in the present study on VRE were examined. Based on their MIC values (Table 2), the antibacterial activities of the isoflavans and the isoflavones, bearing prenyl and phenolic hydroxyl groups, were promising. Our previous study [17] also indicated that compounds with prenyl moieties, such as gancaonin I (**1**), licoarylcoumarin (**19**), and glycycomarin (**21**), showed noticeable anti-VRE effects. Taken together, we conclude that licorice phenolics, particularly those with prenyl moieties, could be used for the development of anti-VRE agents. The mechanisms of action of these phenolics as well as their potential synergistic effects remain to be clarified and the possibility of presence of potential synergistic effects between these identified licorice constituents are remained to be clarified. With regard to their promising antibiotic activities, the phenolic constituents from licorice could be used as lead compounds for developing new antibacterial agents.

## Acknowledgments

This study was supported in part by Drug Discovery Project for Intractable Infectious Diseases of Okayama University (IIDPO). Eerdunbayaer thanks Jinghao Qi, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, for his helpful advice. The NMR instrument used is the property of the Advanced Science Research Center, Okayama University.

## Author Contributions

The contributions of the respective authors are as follows: Eerdunbayaer performed isolation, identification, and structure elucidation of the constituents, and prepared the manuscript. M. A. A. Orabi contributed to checking and confirming all of the procedures of the isolation and structural identification, especially interpretation of the NMR spectra, and also to preparing the manuscript. H. Aoyama contributed to the MS measurements and interpretation of those spectra. T. Kuroda contributed to the antibacterial experiments. This study was performed based on the planning of T. Hatano, the corresponding author.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

1. Horiuchi, K.; Shiota, S.; Hatano, T.; Yoshida, T.; Kuroda, T.; Tsuchiya, T. Antimicrobial activity of oleanolic acid from *Salvia officinalis* and related compounds on vancomycin-resistant *Enterococci* (VRE). *Biol. Pharm. Bull.* **2007**, *30*, 1147–1149.
2. Orsi, G.B.; Ciorba, V. Vancomycin resistant *Enterococci* healthcare associated infections. *Ann. Ig.* **2013**, *25*, 485–492.
3. Rice, L.B. Emergence of vancomycin-resistant *Enterococci*. *Emerg. Infect. Dis.* **2001**, *7*, 183–187.
4. McNeil, S.A.; Clark, N.M.; Chandrasekar, P.H.; Kauffman, C.A. Successful treatment of vancomycin-resistant *Enterococcus faecium* bacteremia with linezolid after failure of treatment with synergid (quinupristin/dalfopristin). *Clin. Infect. Dis.* **2000**, *30*, 403–404.
5. Isbrucker, R.A.; Burdock, G.A. Risk and safety assessment on the consumption of licorice root (*Glycyrrhiza* sp.), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of glycyrrhizin. *Regul. Toxicol. Pharmacol.* **2006**, *46*, 167–192.
6. Shen, X.-P.; Xiao, P.-G.; Liu, C.-X. Research and application of *Radix Glycyrrhizae*. *Asian J. Pharmacodyn. Pharmacokinet.* **2007**, *7*, 181–200.
7. Asl, M.N.; Hosseinzadeh, H. Review of pharmacological effects of *Glycyrrhiza* sp. and its bioactive compounds. *Phytother. Res.* **2008**, *22*, 709–724.
8. Messier, C.; Epifano, F.; Genovese, S.; Grenier, D. Licorice and its potential beneficial effects in common oro-dental diseases. *Oral Dis.* **2012**, *18*, 32–39.
9. Villinski, J.R.; Bergeron, C.; Cannistra, J.C.; Gloer, J.B.; Coleman, C.M.; Ferreira, D.; Gafner, S. Pyrano-isoflavans from *Glycyrrhiza uralensis* with antibacterial activity against *Streptococcus mutans* and *Porphyromonas gingivalis*. *J. Nat. Prod.* **2014**, *77*, 521–526.
10. Gafner, S.; Bergeron, C.; Villinski, J.R.; Godejohann, M.; Kessler, P.; Cardellina, J.H.; Grenier, D. Isoflavonoids and coumarins from *Glycyrrhiza uralensis*: Antibacterial activity against oral pathogens and conversion of isoflavans into isoflavan-quinones during purification. *J. Nat. Prod.* **2011**, *74*, 2514–2519.
11. He, J.; Chen, L.; Heber, D.; Shi, W.; Lu, Q.Y. Antibacterial compounds from *Glycyrrhiza uralensis*. *J. Nat. Prod.* **2006**, *69*, 121–124.

12. Fukai, T.; Marumo, A.; Kaitou, K.; Kanda, T.; Terada, S.; Nomura, T. Anti-*Helicobacter pylori* flavonoids from licorice extract. *Life Sci.* **2002**, *71*, 1449–1463.
13. Irani, M.; Sarmadi, M.; Bernard, F. Leaves antimicrobial activity of *Glycyrrhiza glabra*. *Iran. J. Pharm. Res.* **2010**, *9*, 425–428.
14. Badr, A.E.; Omar, N.; Badria, F.A.A. Laboratory evaluation of the antibacterial and cytotoxic effect of liquorice when used as root canal medicament. *Int. Endod. J.* **2011**, *44*, 51–58.
15. Fukai, T.; Oku, Y.; Hano, Y.; Terada, S. Antimicrobial activities of hydrophobic 2-arylbenzofurans and an isoflavone against vancomycin-resistant *Enterococci* and methicillin-resistant *Staphylococcus aureus*. *Planta Med.* **2004**, *70*, 685–687.
16. Hatano, T.; Shintani, Y.; Aga, Y.; Shiota, S.; Tsuchiya, T.; Yoshida, T. Phenolic constituents of licorice. VIII. Structures of glicophenone and glicoisoflavanone, and effects of licorice phenolics on methicillin-resistant *Staphylococcus aureus*. *Chem. Pharm. Bull.* **2000**, *48*, 1286–1292.
17. Eerdunbayaer; Orabi, M.A.; Aoyama, H.; Kuroda, T.; Hatano, T. Structures of two new flavonoids and effects of licorice phenolics on vancomycin-resistant *Enterococcus* species. *Molecules* **2014**, *19*, 3883–3897.
18. Fukai, T.; Toyono, M.; Nomura, T. On the structure of licoricidin. *Heterocycles* **1988**, *27*, 2309–2313.
19. Tahara, S.; Ingham, J.L.; Mizutani, J. Metabolites of 7-*O*-methyllyuteone from *Botrytis cinerea*. *Nippon Nogeikagaku Kagaku Kaishi* **1989**, *63*, 999–1007.
20. Zeng, L.; Fukai, T.; Nomura, T.; Zhang, R.Y.; Lou, Z.C.; Fukai, T.; Nomura, T. Five new isoprenoid-substituted flavonoids, glyasperins F, G, H, I, and J from the roots of *Glycyrrhiza aspera*. *Heterocycles* **1992**, *34*, 1813–1828.
21. O'Neill, M.J.; Adesanya, S.A.; Roberts, M.F.; Inez, R.P. Inducible isoflavonoids from the lima bean, *Phaseolus lunatus*. *Phytochemistry* **1986**, *25*, 1315–1322.
22. Sil Lee, Y.; Ha Kim, S.; Kyu Kim, J.; Shin, H.K.; Kang, Y.H.; Park, Y.; Lim, S.S. Rapid identification and preparative isolation of antioxidant components in licorice. *J. Sept. Sci.* **2010**, *33*, 664–671.
23. Kiuchi, F.; Chen, X.; Tsuda, Y. Four new phenolic constituents from licorice (root of *Glycyrrhiza* sp.). *Heterocycles* **1990**, *31*, 629–636.
24. Sasaki, H.; Kashiwada, Y.; Shibata, H.; Takaishi, Y. Prenylated flavonoids from the roots of *Desmodium caudatum* and evaluation of their antifungal activity. *Planta Med.* **2012**, *78*, 1851–1856.
25. Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* **1988**, *36*, 2090–2097.
26. Hatano, T.; Yasuhara, T.; Fukuda, T.; Noro, T.; Okuda, T. Phenolic constituents of licorice. II. Structures of licopyranocoumarin, licoaryl coumarin and glicoflavone, and glicoflavone, and inhibitory effects of licorice phenolics on xanthine oxidase. *Chem. Pharm. Bull.* **1989**, *37*, 3005–3009.
27. Kwon, H.J.; Kim, H.H.; Ryu, Y.B.; Kim, J.H.; Jeong, H.J.; Lee, S.W.; Lee, W.S. *In vitro* anti-rotavirus activity of polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*. *Bioorg. Med. Chem.* **2010**, *18*, 7668–7674.

28. Hatano, T.; Takagi, M.; Ito, H.; Yoshida, T. Phenolic constituents of liquorice. VII. A new chalcone with a potent radical scavenging activity and accompanying phenolics from liquorice. *Chem. Pharm. Bull.* **1997**, *45*, 1485–1492.
29. Li, S.; Li, W.; Wang, Y.; Asada, Y.; Koike, K. Prenyl flavonoids from *Glycyrrhiza uralensis* and their protein tyrosine phosphatase-1B inhibitory activities. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5398–5401.
30. Singhal, A.K.; Sharma, R.P.; Thyagarajan, G.; Herz, W.; Govindan, S.V. New prenylated isoflavones and a prenylated dihydroflavonol from *Millettia pachycarpa*. *Phytochemistry* **1980**, *9*, 929–934.
31. Zhang, Q.; Ye, M. Chemical analysis of the Chinese herbal medicine Gan-Cao (licorice). *J. Chromatogr. A* **2009**, *1216*, 1954–1969.
32. Chen, X.J.; Zhao, J.; Meng, Q.; Li, S.P.; Wang, Y.T. Simultaneous determination of five flavonoids in licorice using pressurized liquid extraction and capillary electrochromatography coupled with peak suppression diode array detection. *J. Chromatogr. A* **2009**, *1216*, 7329–7335.
33. Liang, X.; Zhang, L.; Zhang, X.; Dai, W.; Li, H.; Hu, L.; Zhang, W. Qualitative and quantitative analysis of traditional Chinese medicine Niu Huang Jie Du Pill using ultra performance liquid chromatography coupled with tunable UV detector and rapid resolution liquid chromatography coupled with time-of-flight tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2010**, *51*, 565–571.
34. Seo, C.S.; Lee, J.A.; Jung, D.; Lee, H.Y.; Lee, J.K.; Ha, H.; Shin, H.K. Simultaneous determination of liquiritin, hesperidin, and glycyrrhizin by HPLC-photodiode array detection and the anti-inflammatory effect of Pyungwi-san. *Arch. Pharm. Res.* **2011**, *34*, 203–210.
35. Wen, J.; Qiao, Y.; Yang, J.; Liu, X.; Song, Y.; Liu, Z.; Li, F. UPLC-MS/MS determination of paeoniflorin, naringin, naringenin and glycyrrhetic acid in rat plasma and its application to a pharmacokinetic study after oral administration of SiNiSan decoction. *J. Pharm. Biomed. Anal.* **2012**, *66*, 271–277.
36. Zhou, S.; Cao, J.; Qiu, F.; Kong, W.; Yang, S.; Yang, M. Simultaneous determination of five bioactive components in radix glycyrrhizae by pressurised liquid extraction combined with UPLC-PDA and UPLC/ESI-QTOF-MS confirmation. *Phytochem. Anal.* **2013**, *24*, 527–533.

*Sample Availability:* Samples of all of the compounds are unavailable.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (<http://creativecommons.org/licenses/by/3.0/>).