Identification of Antimicrobial and Antioxidant Constituents from Licorice of Russian and Xinjiang Origin

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The organic extracts of two licorices, known in commerce as Russian and Xinjiang licorices, exhibited potent antimicrobial and antioxidant activity. The bioassay-directed chemical investigation of both licorices revealed glabrene, glabridin, and licochalcones A and B as active principles.

Keywords: Glycyrrhiza glabra; Glycyrrhiza inflata; Leguminosae; antimicrobial activity; antioxidant activity; licorice; glabrene; glabridin; licochalcone A; licochalcone B

In a previous paper‡1 we reported the isolation of antimicrobial and antioxidant principles from Chinese licorice known in commerce as xibei licorice (西北甘草, seihoku kanzo in Japanese). In a continuation of this program, licorices of other origin that have medicinal value in this country were also examined for antimicrobial and antioxidant activity. It was found that extracts of Russian and Xinjiang licorices (新薬甘草, shinkyo kanzo in Japanese), which are assigned as roots of Glycyrrhiza (G.) glabra L. var. glandulifera and G. inflata respectively, exhibited reproducible antimicrobial and antioxidant activities. Though these two licorices also contain glycyrrhizin and its analogues as the main constituents, previous chemical investigation‡2 revealed a marked difference in phenolic constituents with relatively low polarity, which are expected to participate in the antimicrobial and antioxidant activities of licorice extracts. Thus, systematic bioassay-directed fractionation of licorice extracts was undertaken in order to identify the active principles, and this paper describes the results.

Results and Discussion

The separation of the bioactive principles from both licorice roots was carried out with the guidance of antimicrobial (against Staphylococcus aureus) and antioxidant activity assays as mentioned in the experimental section. Both the antimicrobial and antioxidant activities were overlapping in most chromatographic fractions, and were not separated.

Two constituents, tentatively named compounds I and II, were obtained as potent antimicrobial and antioxidant principles from Russian licorice. From the proton nuclear magnetic resonance (1H-NMR) spectrum of compound I, the presence of a chromene ring was suggested by the signals of gem-dimethyls [δ 1.38 (6H, s)] and an AB system of olefinic protons [δ 5.64 (1H, d, J = 9.9 Hz), 6.55 (1H, d, J = 9.9 Hz)]. The presence of an olefinic proton [δ 6.53 (1H, s)] and an allylic methylene [δ 4.90 (2H, s)], which characterizes the hetero ring of an isoflavene, was also apparent. These findings readily led to the assignment of compound I as the structure 1, which was confirmed by direct comparison (mixed melting point, infrared (IR) and NMR spectra) with authentic glabrene.‡3 Compound II also show-

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Licochalcone A</th>
<th>Licochalcone B</th>
<th>MIC (µg/ml) Glabrene</th>
<th>Glabridin</th>
<th>Streptomycin</th>
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<tbody>
<tr>
<td>Gram-positive bacteria</td>
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<tr>
<td>Staphylococcus aureus</td>
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<td>31.3</td>
<td>7.81</td>
<td>1.95</td>
<td>1.95</td>
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<tr>
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<td>3.91</td>
<td>31.3</td>
<td>7.81</td>
<td>3.91</td>
<td>1.95</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
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<tr>
<td>Escherichia coli</td>
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<td>&gt; 250</td>
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<td>7.81</td>
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<tr>
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<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>31.3</td>
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<td>&gt; 250</td>
<td>15.6</td>
<td>7.81</td>
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<tr>
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<td>&gt; 250</td>
<td>31.3</td>
<td>7.81</td>
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<tr>
<td>Fungi</td>
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<tr>
<td>Microsporum giselesi</td>
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<td>&gt; 250</td>
<td>15.6</td>
<td>3.91</td>
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<tr>
<td>Aspergillus niger</td>
<td>&gt; 250</td>
<td>&gt; 250</td>
<td>31.3</td>
<td></td>
<td>&gt; 250</td>
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</table>

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ed the signals due to the chromene ring in addition to an ABMXX system characteristic of the C2-C8 portion of the isoflavon moiety [δ 2.84 (1H, dd, J = 15.8, 4.3 Hz), 2.96 (1H, dd, J = 15.8, 10.7 Hz), 3.47 (1H, m), 4.01 (1H, dd, J = 10.1, 1.1 Hz), 4.36 (1H, br d, J = 10.1 Hz)] in the 1H-NMR spectrum. It was found to be identical with glabridin (mixed melting point, IR and NMR spectra) in direct comparison with an authentic sample. 21

The extracts of Xinjiang licorice furnished two pigments as active principles, tentatively named compounds III and IV. The ultraviolet (UV) and IR spectra of compounds III and IV were indicative of the chalcone skeleton. The presence of a 1,1-dimethyl-2-butene moiety in compound III was suggested by the following signals in the 1H-NMR spectrum [δ 1.44 (6H, s), 5.32 (1H, d, J = 11.3 Hz), 5.36 (1H, d, J = 18 Hz), 6.19 (1H, dd, J = 18, 11.3 Hz)], while compound IV showed no signals assignable to the C6 unit. A pronounced [M - 31]+ fragment peak was seen in the mass spectra (MS) of both chalcones, which is characteristic of a retro-chalcone possessing a methoxy group at the 2-position. 21 Thus, the structures of compounds III and IV were characterized as 3 and 4, respectively, and the compounds were identified with licochalcone A and B by mixed melting point determination, and comparisons of IR and NMR spectra. 21

Table I illustrates the antimicrobial activity spectra of these four constituents against various types of microorganisms including bacteria, yeasts and fungi. All of these compounds inhibited the growth of gram-positive bacteria such as Staphylococcus aureus and Bacillus subtilis. In particular, the potencies of the antimicrobial activity of licochalcone A and glabridin were comparable to that of a well-known antibiotic, streptomycin. On the other hand, none of them was active against gram-negative bacteria. However, there were remarkable differences in the antimicrobial spectra of these compounds. For example, glabridin, a major phenolic constituent of Russian licorice, also exhibited significant growth-inhibitory activity against yeasts and fungi, against which the other three compounds as well as streptomycin were inactive. Mitscher et al. also reported the isolation of glabridin, glabrene and some minor phenolic constituents from the root of G. glabra var. typica (Spanish licorice) as antimicrobial principles against some bacterial strains. 31

The antioxidant activities of the compounds isolated here are shown in Fig. 1. Both licochalcone A and B exhibited potent antioxidant activity comparable to that of vitamin E as determined by the active oxygen method. Glabrene showed the most potent activity among the compounds isolated here, being three times as potent as vitamin E, while glabridin exhibited no significant activity in spite of its structural similarity to glabrene. Glabrene easily undergoes rapid autoxidation to form a complex colored gum in acetone solution bubbled with air, while glabridin is resistant to oxidation by molecular oxygen. Okuda et al. reported the radical scavenging effect of licochalcone A and B (from Xinjiang licorice), and glycyrrhizin (from xibei licorice), 41 but according to the active oxygen method the latter compound was inactive. 11 This indicates that the antioxidant activities of the compounds described here are not necessarily due to their radical scavenging effect.

The phenolic constituents in licorice have not drawn as much attention as glycyrrhizin and its analogues, which have found considerable commercial value as therapeutic agents and sweeteners. The only exception is glycyrrhizin, a glycyrrhizin-free fraction prepared from the licorice extract, and considered to abound in phenolic constituents, which was proved to have anti-ulcer activity. 51 Since the content of glabridin, glabrene, and licochalcone A and B in each licorice were estimated at 9.1%, 2.8%, 18.2% and 2.7%, respectively, by high-performance liquid chromatography (HPLC), these compounds should account for much of the antimicrobial and antioxidant activities of licorice extracts. The presence of potent antimicrobial and antioxidant principles in licorice in significant content may further supplement its value not only as a crude drug but also as a food additive.

Experimental

All melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Spectral data were obtained using the following apparatus: 1H-NMR and carbon-13 nuclear magnetic resonance (13C-NMR) spectra with JEOL JMN GX-400 (400 MHz) spectrometers with tetramethylsilane as an internal standard; MS with a JEOL JMS-D300 mass spectrometer; IR spectra with a Hitachi 215 grating infrared spectrometer; UV spectra with a Shimadzu UV 240 spectrophotometer. Silica gel column chromatography was carried out on Wakogel C-200 with CHCl3-MeOH mixture, and reverse-phase silica gel column chromatography on YMC Gel ODS 120A (Yamamura Kagaku) with MeOH-2% AcOH (3:2). The HPLC analyses were carried out on a JASCO TWINCLE apparatus.

Plant Materials Licorice roots used in this study were obtained from Maruzen Kasei Co., Ltd. (Onomichi, Japan).

Assay Procedure for Antioxidant Activity The antioxidant activity of test samples was evaluated based on the active oxygen method as described in the previous paper, except that each sample was tested at the concentration of 200 ppm.

Determination of Minimal Inhibitory Concentration (MIC) MICs of test samples were determined as described previously. 11 Strains of microorganisms used in this experiment were as follows. Staphylococcus aureus IFO 3060, Bacillus subtilis IFO 1668, Escherichia coli IFO 3366, Pseudomonas aeruginosa JCM 2776, Saccharomyces cerevisiae IFO 9306, Candida utilis IFO 1086, Mucor pusillus HUT 1186, Aspergillus niger IFO

![Fig. 1. Antioxidant Activity of Constituents Isolated from Russian and Xinjiang Licorice](image-url)
4407. Each microorganism was cultured in the usual manner.\textsuperscript{1)}

**Isolation of Constituents from Russian Licorice** The Russian licorice (1 kg) was extracted with CH\textsubscript{2}Cl\textsubscript{2} to give the extract (29 g). The extract was chromatographed over silica gel to give fractions (frs.) 1—6. Fraction 5, where much of both activities occurred, was rechromatographed on a reverse-phase silica gel column to give ten fractions. Recrystallization of fr. 3, which showed the most potent activities, from benzene-Me\textsubscript{2}CO furnished 0.1 g of pure glabrene (1). Fraction 5 was also recrystallized from benzene-hexane to afford 0.3 g of glabridin (2) in pure form.

**Glabrene (1)** Pale yellow plates from benzene-Me\textsubscript{2}CO, mp 190—193°C (dec.). UV \( \lambda_{\text{max}}^{\text{nm}} \) 245, 283, 293, 321. IR \( \nu_{\text{max}}^{\text{cm}^{-1}} \): 3320, 2970, 1635, 1610, 1598, 1500, 1435. \( ^{1}H\)-NMR (400 MHz, DMSO-d\textsubscript{6}) \( \delta \): 1.38 (6H, s), 4.90 (2H, br s), 5.64 (1H, d, \( J = 9.9 \) Hz), 6.26 (1H, d, \( J = 2.6 \) Hz), 6.35 (1H, d, \( J = 8.1 \) Hz), 6.41 (1H, d, \( J = 8.4 \) Hz), 6.53 (1H, s), 6.55 (1H, d, \( J = 9.9 \) Hz), 6.93 (1H, d, \( J = 8.1 \) Hz), 7.01 (1H, d, \( J = 8.4 \) Hz). EI-MS m/z (relative intensity, %): 338 (M\textsuperscript{+}, 24), 307 (M\textsuperscript{+}−OMe, 100).

**Glabridin (2)** Colorless plates from benzene-hexane, mp 165—167°C. UV \( \lambda_{\text{max}}^{\text{nm}} \) 228, 282, 312. IR \( \nu_{\text{max}}^{\text{cm}^{-1}} \): 3300, 2970, 1635, 1610, 1582, 1480. \( ^{1}H\)-NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \): 1.40, 1.42 (3H each, s), 2.84 (1H, dd, \( J = 1.58, 4.3 \) Hz), 2.96 (1H, dd, \( J = 15.8, 10.7 \) Hz), 3.47 (1H, m), 4.01 (1H, dd, \( J = 10.1, 10.1 \) Hz), 4.36 (1H, br d, \( J = 10.1 \) Hz), 5.03, 5.13 (1H each, br s, 2 × OH), 5.56 (1H, d, \( J = 10.1 \) Hz), 6.27 (1H, d, \( J = 2.4 \) Hz), 6.36 (1H, d, \( J = 8.2, 2.4 \) Hz), 6.37 (1H, d, \( J = 8.4 \) Hz), 6.44 (1H, d, \( J = 10.1 \) Hz), 6.81 (1H, d, \( J = 8.2 \) Hz), 6.93 (1H, d, \( J = 8.4 \) Hz). EI-MS m/z (relative intensity, %): 324 (M\textsuperscript{+}, 5), 255 (M\textsuperscript{+}−OMe, 100).

**Licochalcone A (3)** Yellow needles from MeOH–H\textsubscript{2}O, mp 99—100°C. UV \( \lambda_{\text{max}}^{\text{nm}} \) 254, 312, 377. IR \( \nu_{\text{max}}^{\text{cm}^{-1}} \): 3250, 1620. \( ^{1}H\)-NMR (100 MHz, CDCl\textsubscript{3}) \( \delta \): 1.44 (6H, s), 3.83 (3H, s), 5.32 (1H, d, \( J = 11.3 \) Hz), 5.36 (1H, d, \( J = 18 \) Hz), 6.19 (1H, dd, \( J = 18, 11.3 \) Hz), 6.44 (1H, d, \( J = 8.2 \) Hz), 7.45 (1H, s), 7.60 (1H, d, \( J = 15.6 \) Hz), 7.98 (2H, d, \( J = 8.2 \) Hz), 8.03 (1H, d, \( J = 15.6 \) Hz). EI-MS m/z (relative intensity, %): 338 (M\textsuperscript{+}, 24), 307 (M\textsuperscript{+}−OMe, 100).

**Licochalcone B (4)** Yellow needles from MeOH–H\textsubscript{2}O, mp 196—197°C. UV \( \lambda_{\text{max}}^{\text{nm}} \) 308, 364. IR \( \nu_{\text{max}}^{\text{cm}^{-1}} \): 3250, 1620. \( ^{1}H\)-NMR (100 MHz, Me\textsubscript{2}CO-d\textsubscript{6}) \( \delta \): 3.89 (3H, s), 6.73 (1H, d, \( J = 8.5 \) Hz), 6.98 (2H, d, \( J = 8.5 \) Hz), 7.31 (1H, d, \( J = 8.5 \) Hz), 7.31 (1H, d, \( J = 8.5 \) Hz), 7.69 (1H, d, \( J = 16 \) Hz), 8.03 (1H, d, \( J = 16 \) Hz). EL-MS m/z (relative intensity, %): 326 (M\textsuperscript{+}, 5), 255 (M\textsuperscript{+}−OMe, 100).

**Quantitative Analysis of 1—4 in Licorice by HPLC** The CH\textsubscript{2}Cl\textsubscript{2} extract of each licorice was analyzed by HPLC under the following conditions: column, µ-Bondapak C-18; eluent, CH\textsubscript{3}CN–2% AcOH (45:55); detector, UV 336 nm; flow rate, 2 ml/min. Column temperature, 40°C.

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**References**


