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 licorice (新疆甘草, 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 (^{1}H-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.[2] Compound II also show-

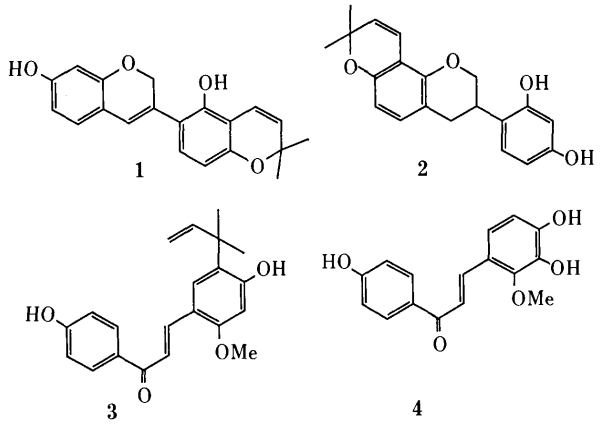

TABLE I. Antimicrobial Activity of Constituents of Licorice

Microorganisms	MIC (μg/ml) Licochalcone A	Licochalcone B	Glabrene	Glabridin	Streptomycin
Gram-positive bacteria					
Staphylococcus aureus	1.95	31.3	7.81	1.95	1.95
Bacillus subtilis	3.91	31.3	7.81	3.91	1.95
Gram-negative bacteria					
Escherichia coli	>250	>250	>250	>250	7.81
Pseudomonas aeruginosa	>250	>250	>250	>250	31.3
Yeasts					
Saccharomyces cerevisae	>250	>250	15.6	7.81	>250
Candida utilis	>250	>250	31.3	7.81	>250
Fungi					
Mucro pusillus	>250	>250	15.6	3.91	>250
Aspergillus niger	>250	>250	>250	31.3	>250

ed the signals due to the chromene ring in addition to an ABMXX' system characteristic of the C_2–C_4 portion of the isoflavan 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, 10.1 Hz), 4.36 (1H, br d, J=10.1 Hz)] in the ^{1}H-NMR spectrum. It was found to be identical with glabridin (mixed melting point, IR and NMR spectra) in direct comparison with an authentic sample.[2]

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 ^{1}H-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 C_5 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.[2] Thus, the structures of compounds III and IV were characterized as **3** and **4**, respectively, and the compounds were identified with licochalcones A and B by mixed melting point determination, and comparisons of IR and NMR spectra.[2]

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.[3]

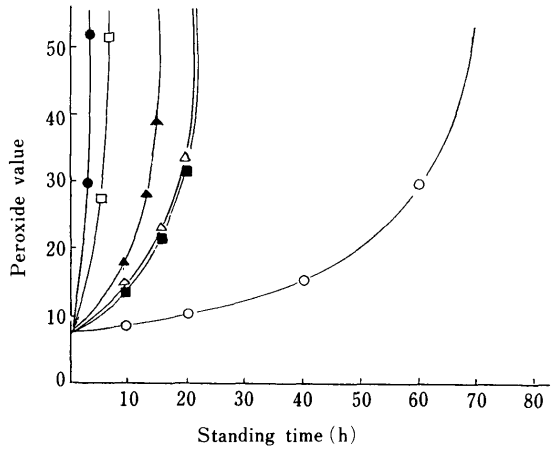

Fig. 1. Antioxidant Activity of Constituents Isolated from Russian and Xinjiang Licorice

The peroxide value (POV) is the quantitative value of peroxide formed in lard by interaction with active oxygen. When POV is plotted against time (h), it is usually found that the curve starts rising abruptly at some time point. Until then the formation of peroxide is inhibited. Thus the potency of each sample as an antioxidant is expressed by the span of time when POV reaches this point. The procedure to measure POV were described in the experimental part or in the previous paper.[1] Each sample was tested for antioxidant activity at the concentration of 200 ppm. —●—, blank; —■—, licochalcone A; —▲—, licochalcone B; —○—, glabrene; —□—, glabridin; —△—, vitamin E.

The antioxidant activities of the compounds isolated here are shown in Fig. 1. Both licochalcones 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 grabridin 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 licochalcones A and B (from Xinjiang licorice), and glycycoumarin (from xibei licorice),[4] but according to the active oxygen method the latter compound was inactive.[1] 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 F_{M100}, a glycyrrhizin-free fraction prepared from the licorice extract, and considered to abound in phenolic constituents, which was proved to have anti-ulcer activity.[5] Since the content of glabridin, glabrene, and licochalcones 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: ^{1}H-NMR and carbon-13 nuclear magnetic resonance (^{13}C-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 $CHCl_3$–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.[1]

Determination of Minimal Inhibitory Concentration (MIC) MICs of test samples were determined as described previously.[1] 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 0306, *Candida utilis* IFO 1086, *Mucor pusillus* HUT 1186, *Aspergillus niger* IFO

4407. Each microorganism was cultured in the usual manner.[1]

Isolation of Constituents from Russian Licorice The Russian licorice (1 kg) was extracted with CH_2Cl_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 reversephase silica gel column to give ten fractions. Recrystallization of fr. 3, which showed the most potent activities, from benzene–Me_2CO 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_2CO, mp 190—193 °C (dec.). UV λ_{max}^{EtOH} nm: 245, 283, 293, 321. IR ν_{max}^{KBr} cm^{-1}: 3320, 2970, 1635, 1610, 1598, 1500, 1435. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 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, 2.6 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: 322 (M^+).

Glabridin (2) Colorless plates from benzene–hexane, mp 165—167 °C. UV λ_{max}^{EtOH} nm: 228, 282, 312. IR ν_{max}^{KBr} cm^{-1}: 3300, 2970, 1635, 1610, 1582, 1480. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 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, dd, J=8.2, 2.4 Hz), 6.37 (1H, d, J=8.4 Hz), 6.64 (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: 324 (M^+).

Isolation of Constituents from Xinjiang Licorice The Xinjiang licorice (1 kg) was extracted with MeOH to give the extract (180 g), which was further partitioned between $CHCl_3$ and H_2O. The organic layer was concentrated *in vacuo* and chromatographed over silica gel to give four fractions. The fraction showing the most potent activities was rechromatographed over reverse-phase silica gel to give frs. 1—7. Fractions 4 and 5 were combined and recrystallized from MeOH–H_2O to give licochalcone A (1.54 g). The water layer was extracted with ethyl acetate, and the organic layer was concentrated under reduced pressure. The residue was chromatographed over silica gel to give four fractions. Fraction 2 was separated by a reverse-phase silica gel chromatography to give frs. 1—8. Recrystallization of frs. 2 and 3 from MeOH–H_2O furnished licochalcone B (0.45 g).

Licochalcone A (3) Yellow needles from MeOH–H_2O, mp 99—100 °C. UV λ_{max}^{EtOH} nm: 254, 312, 377. IR ν_{max}^{KBr} cm^{-1}: 3250, 1620. ^{1}H-NMR (100 MHz, $CDCl_3$) δ: 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, s), 6.98 (2H, 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^+, 24), 307 (M^+ − OMe, 100).

Licochalcone B (4) Yellow needles from MeOH–H_2O, mp 196—197 °C. UV λ_{max}^{EtOH} nm: 308, 364. IR ν_{max}^{KBr} cm^{-1}: 3250, 1620. ^{1}H-NMR (100 MHz, Me_2CO-d_6) δ: 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 (d, J=8.5 Hz), 7.69 (1H, d, J=16 Hz), 8.03 (1H, d, J=16 Hz). EI-MS m/z (relative intensity, %): 286 (M^+, 5), 255 (M^+ − OMe, 100).

Quantitative Analysis of 1—4 in Licorice by HPLC The CH_2Cl_2 extract of each licorice was analyzed by HPLC under the following conditions: column, μ-Bondapak C-18; eluent, CH_3CN–2% AcOH (45 : 55); detector, UV 336 nm; flow rate, 2 ml/min. Column temperature, 40 °C.

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