Chapter 4

Licorice as a Resource for Pharmacologically Active Phenolic Substances: Antioxidant and Antimicrobial Effects

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Additional information is available at the end of the chapter

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Abstract

The findings from our studies on licorice phenolics are summarized here. The following types of flavonoids, i.e., flavones, flavonols, flavanones, chalcones, isoflavones, isoflavanones, isoflavans, 3-arylcoumarins, coumestans, pterocarpans, 2-benzyldihydrobenzofuran-3-ones, benzyl phenyl ketones, 2-arylbenzofurans, and others, were identified by the structural studies. Among them, licochalcone A (chalcone), isolicoflavonol (flavonol), glycycoumarin (3-arylcoumarin), and glycyrrhisoflavone (isoflavone) displayed antihuman immunodeficiency virus effects, and also 8-(γ,γ-dimethylallyl)-wighteone (isoflavone) and 3′-(γ,γ-dimethylallyl)-kievitone (isoflavanone) showed potent antibacterial effects on methicillin-resistant *Staphylococcus aureus* (MRSA) strains. Licoricidin (isoflavan) suppressed the oxacillin resistance of the MRSA strains noticeably. Effects of phenolics with related structures isolated from *Psoralea corylifolia* were also examined, and bakuchiol (meroterpene), isobavachalcone, and corylifol B (chalcones) also showed potent effects on MRSA strains. Some licorice phenolics such as licoricidin (isoflavan), 8-(γ,γ-dimethylallyl)-wighteone (isoflavone), and gancaonin I (2-arylbenzofuran) also showed potent antibacterial effects on vancomycin-resistant *Enterococcus* (VRE) strains. The potency of the effects largely depended on their structures including the lipophilic prenyl or related substituents and also phenolic hydroxyl groups. Inhibitory effects of licorice phenolics on oxidative enzymes, in addition to their radical-scavenging effects, are also shown. The methods used in the structural studies and high-performance liquid chromatographic analysis of licorice extracts are described shortly, too.

Keywords: licorice, *Glycyrrhiza*, *Psoralea*, flavonoid, antimicrobial effect

1. Introduction

Licorice (liquorice), the underground portion of *Glycyrrhiza* species, has been used as a remedy for various types of stress, inflammatory diseases, digestive organ disorders, and pain in traditional medicine in Asian and European countries [1, 2]. The main constituent, glycyrrhizin, and the associated aglycone, glycyrrhetinic acid, are also used in modern medicine, whereas the phenolic constituents have been implicated in promoting improved health, particularly with regard to stomach ulcers [2]. Therefore, several research groups have investigated the phenolic constituents of licorice [3] and found that it has beneficial effects for health, including antimicrobial properties [4, 5]. In this chapter, we summarize our studies on phenolic constituents and some of their pharmacological effects, including those linked to drug-resistant bacteria.

2. Findings from our research

Our research on licorice constituents began with an investigation of tannin-like substances in licorice, because tannins and related constituents in medicinal plants have remarkable antioxidant effects, in addition to their fundamental property of binding with proteins, which is related to its various pharmacological effects [6–8]. In fact, licorice extracts of various origins contain tannin-like substances and show protein-binding properties [9]; our additional studies revealed that some phenolic constituents related to flavonoids contribute to this property. Therefore, we investigated these flavonoids and related compounds as discussed below.

2.1. Purification of licorice phenolics

Although classic column chromatography using silica gel has been applied to the separation of phenolic plant constituents, the irreversible adsorption of phenolic constituents (particularly, tannins or tannin-like substances) has limited ability to effectively separate these compounds. Because countercurrent distribution (CCD) does not use solid supports for separation, it can be applied to solve the problem of irreversible adsorption. Thus, centrifugal partition chromatography (CPC) and droplet countercurrent chromatography (DCCC), which were devised as effective methods for CCD, in addition to simple CCD using separatory funnels, were applied to purify the licorice phenolics in our studies. The solvent system chloroform-methanol-water (7:13:8, by volume) was primarily used for the separation of licorice phenolics derived from *Glycyrrhiza inflata* [9, 10] and those derived from *G. uralensis* [11–16] in these CCD processes. Combinations of column chromatography on a silica gel, ODS-gel, and/or polystyrene gel (MCI-gel CHP-20P) with CCD also afforded satisfactory separation [17, 18]. High-performance liquid chromatography (HPLC) was applied for final purification and to establish the purity of the isolated compounds [18, 19]. However, the CCD systems using the solvent systems ethyl acetate-*n*-propanol-water, *n*-hexane-ethanol-water-ethyl acetate, and chloroform-methanol-*n*-propanol-water, in addition to chloroform-methanol-water, were also useful for separating various types of phenolic constituents [17, 20].

2.2. Structural study on licorice phenolics exploring the diversity of their skeletons

Although the structures of aforementioned licorice phenolics were characterized based on the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra, including various 1D and 2D methods, the following spectroscopy methods were also key in establishing the structures. Electron impact mass spectrometry (EI-MS) is a useful method for obtaining structural information using fragment ions [16]. On the other hand, fast-atom bombardment (FAB) and electrospray ionization mass spectrometry (ESI-MS) are applicable to the ionization of phenolics, including phenolic glycosides. Notably, the high-resolution FAB and ESI-MS have been used to determine their molecular formulae [17]. Ultraviolet-visible (UV-Vis) spectroscopy was useful for discriminating between phenolic skeletons even if the ^{1}H NMR spectra were quite similar to each other, as was the case for 3-arylcoumarins and the corresponding isoflavones [16]. Electronic circular dichroism (ECD) spectroscopy was effective not only for identifying the configuration of asymmetric carbons (e.g., those in flavanones, isoflavans, and isoflavanones [9, 15, 17]) in the flavonoid skeletons but also for explaining the spatial relationship between the chromophores in acylated flavonoid glycoside molecules [17]. Based on the data obtained by the aforementioned spectroscopy methods, we uncovered new compound structures and identified known ones isolated from licorice, which can be classified into subgroups based on their structural skeletons as shown in **Table 1**.

As shown in **Table 1**, various types of phenolics have been found in licorice, in addition to the major phenolics (liquiritin, isoliquiritin, and related ones) [21], and their pharmacological properties differ depending on their structures. The strength of the order of the effects also differs depending on the properties examined. Especially, their phenolic hydroxyl and prenyl substituents and also their skeletons related to the molecular flexibility should be considered for their respective properties.

2.3. Properties of licorice phenolics in relation to their health effects

Polyphenols have been linked to antioxidant effects, and some polyphenols such as tannins have protein-binding effects. Interaction of tannins with protein molecules is regarded to be based on hydrophobic interaction and hydrogen bonding and also covalent bonding in some cases [22]. Although some researches focused on the participation of proline residues of proteins in the complexation [23], the modes of complexation are largely dependent on the structures of tannins and proteins/peptides [24–27]. Therefore, further studies using various types of polyphenols should be conducted in order to clarify the complexation. Thus, we examined the binding and antioxidant effects of licorice phenolics.

2.3.1. Protein-binding and antioxidant effects

Among the isolated compounds found in large quantities in licorice materials, licochalcone B from Sinkiang (Xinjiang) licorice (mainly collected in the Xinjiang Uyghur Autonomous Region of China) showed the most potent binding activity with proteins, followed by glycyrrhisoflavone from Si-pei (Xi-bei) licorice [9]. Tannins displayed different binding effects depending upon their structures, and licochalcone B and glycyrrhisoflavone (**Figure 1**) showed

Subgroup	Compounds	Origin [a]
Flavones	4′,7-Dihydroxyflavone [9]	*G. inflata*
	3′,4′,7-Trihydroxyflavone [17]	*G. uralensis*
Flavonols	Isolicoflavonol [9], kaempferol-3-*O*-methyl ether [12], licoflavonol, topazolin [16], kaempferol [18], fisetin, glycyrrhiza-flavonol A * [20]	*G. uralensis*
Flavanones	6″-Acetylliquiritin; naringenin [15]; 3′-prenylnaringenin [16]; licorice-glycosides C1 *, C2 *, D1 *, D2 *, and E *; liquiritin apioside [17]; liquiritigenin; liquiritin [21]	*G. uralensis*
Chalcones	Licochalcones A and B [9]	*G. inflata*
	Echinatin [15]; isoliquiritin apioside; licorice glycosides A * and B *; neoisoliquiritin [17]; licochalcone B; tetrahydroxy methoxychalcone * [20]; isoliquiritigenin; isoliquiritin [21]	*G. uralensis*
Isoflavones	Glycyrrhisoflavone * [9]; glisoflavone * [12]; genistein; glicoricone * [14]; 8-(γ,γ-dimethylallyl)-wighteone; gancaonin G; isoangustone A; isowighteone; semilicoisoflavone B [15]; allolicoisoflavone B; 7-*O*-methylluteone; orobol [16]; glycyroside [17]; 5,7-di-*O*-methylluteone *; 6,8-diprenylorobol; formononetin; licoricone [18]; calycosin; glycyrrhiza-isoflavones A *, B *, and C * [20]	*G. uralensis*
Isoflavanones	Glycyrrhisoflavanone * [9], 3′-(γ,γ-dimethylallyl)-kievitone, glicoisoflavanone *, glyasperin F, licoisoflavanone [15], glisoflavanone * [16], glyasperin J, glyasperin J trimethyl ether [19]	*G. uralensis*
Isoflavans	Glyasperin C, glyasperin D, licoricidin, (3*R*)-vestitol [15], (3*R*)-vestitol-7-*O*-glucoside* [17]	*G. uralensis*
3-Arylcoumarins	Glycycoumarin [9], licopyranocoumarin * [11], licoarylcoumarin * [12], glycerin [15], isoglycycoumarin, licofuranocoumarin * [16], 3-(*p*-hydroxyphenyl)-7-methoxycoumarin [18], isolicopyranocoumarin * [20]	*G. uralensis*
Coumestans	Glycyrol, isoglycyrol [15], isotrifoliol * [16], dimethylglycyrol * [18]	*G. uralensis*
Pterocarpans	Demethylhomopterocarpan [19]	*G. uralensis*
2-Benzyldihydro-benzofuran-3-ones	Carpusin [17]	*G. uralensis*
Benzyl phenyl ketones	Glicophenone *, licoriphenone [15]	*G. uralensis*
2-Arylbenzofurans	Licofuranone * [14], licocoumarone [15], gancaonin I [18], glycybenzofuran, 4′-*O*-methylglycybenzofuran *, neoglycybenzofuran * [19]	*G. uralensis*
Benzoic acids	*p*-Hydroxybenzoic acid [20]	*G. uralensis*

[a]The origins were assigned accordingly: *G. uralensis* [Tong-pei licorice and Si-pei (Xi-bei) licorice] and *G. inflata* [Sinkiang (Xinjiang) licorice].
*New compounds.

Table 1. Classification of isolated licorice phenolics.

binding effects more potent than, or comparable to, those of some hydrolyzable tannins such as pedunculagin or corilagin [9, 28].

Then, we examined phenolic radical-scavenging effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Again, licochalcone B displayed the most potent scavenging effects on the DPPH radicals among the examined compounds; licochalcone A showed weaker effects, and isoliquiritigenin and liquiritigenin had negligible effects. The order of potency was as follows:

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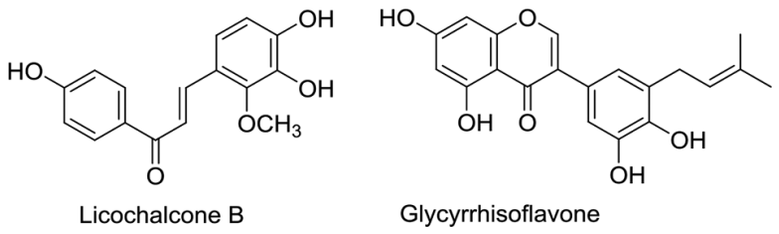

Figure 1. Licorice phenolics with protein-binding activity.

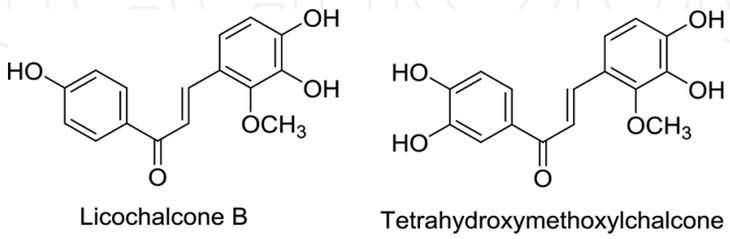

Figure 2. Licorice phenolics forming stable radicals in alkaline DMSO.

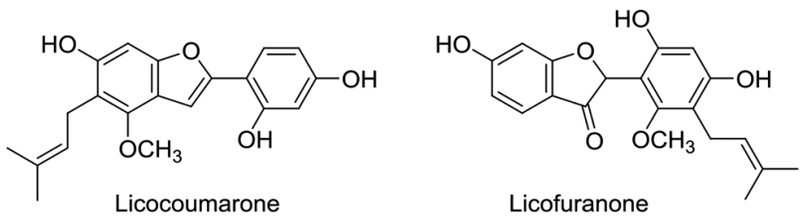

Figure 3. Licorice phenolics with inhibitory effects on oxidative enzymes.

licochalcone B > licochalcone A >> isoliquiritigenin > liquiritigenin. This order of the scavenging effects was the same as that of the reported suppressive effects on lipoxygenase products in arachidonate metabolism [29]. Because stable radical formation was correlated with potent radical-scavenging effects, we examined the formation of radical species from two chalcones, licochalcone B and tetrahydroxy methoxylchalcone (**Figure 2**). As expected, they showed stable electron spin resonance (ESR) signals attributable to their radicals formed by air oxidation in alkaline DMSO solutions [20].

On the other hand, we reported that several licorice phenolics showed inhibitory effects on xanthine oxidase and monoamine oxidase. Licocoumarone, a 2-arylbenzofuran, showed the most potent inhibitory effects on xanthine oxidase, followed by the effects of licochalcone B, licochalcone A, and glycyrrhisoflavone [12]. Two 2-arylbenzofurans, licocoumarone and licofuranone, also showed potent inhibitory effects on monoamine oxidase (**Figure 3**), followed by glycyrrhisoflavone and genistein [14].

The role of xanthine oxidase in the catalysis of the reaction of xanthine into uric acid has been linked to gout and also correlates with the generation of superoxide anion radicals, a reactive oxygen species (ROS). Thus, we examined the effects of licorice phenolics on superoxide generation because ROS have been linked to various kinds of oxidative damage

including human organ damage. Licorice phenolics showed suppressive effects on superoxide anion radical generation, both in the enzymatic and nonenzymatic systems examined. In addition to a combination of xanthine oxidase and xanthine (from the enzymatic system), a combination of phenazine methosulfate (PMS) and a reduced form of nicotinamide adenine dinucleotide hydride (NADH) (from the nonenzymatic system) were used for the generating system. On the other hand, detection of the superoxide anion radical was performed using nitroblue tetrazolium and cytochrome c [30]. Three experimental systems composed of the generating and the detection systems indicated that licochalcone B and glycyrrhisoflavone showed potent suppressing effects on superoxide anion radical generation, which are comparable to those of a specific representative flavonoid (quercetin) and a tannin (pedunculagin).

2.3.2. Antihuman immunodeficiency virus effects and suppressive effects on human immunodeficiency virus promoter activity

Further investigation of licorice phenolics revealed that licochalcone A, isolicoflavonol, glycycoumarin, and glycyrrhisoflavone had antiviral effects on human immunodeficiency virus (HIV) (**Figure 4**). HIV causes a "giant cell" in the infected cells OKM-3T (= OKM-1) due to the cytopathic effects of the virus. The aforementioned compounds had inhibitory effects on giant cell formation of a cell line infected with HIV [11, 30]. The mechanisms underlying these antiviral effects may be different from those observed for tannins [31].

Suppressive effects of licorice phenolics on HIV promoters have also been revealed. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced HIV promoter activity in transfected Jurkat cells was suppressed by glycyrrhisoflavone (isoflavone), tetrahydroxymethoxychalcone, licochalcones A and B (chalcones), glycycoumarin, licopyranocoumarin (3-arylcoumarins), and licocoumarone (2-arylbenzofuran). Although tannins also showed suppressive effects in this experimental system, the effects of licorice phenolics were more potent [32]. On the other hand, licorice phenolics did not show suppressive effects on the cytomegalovirus promoter activity in an analogous experimental system [32].

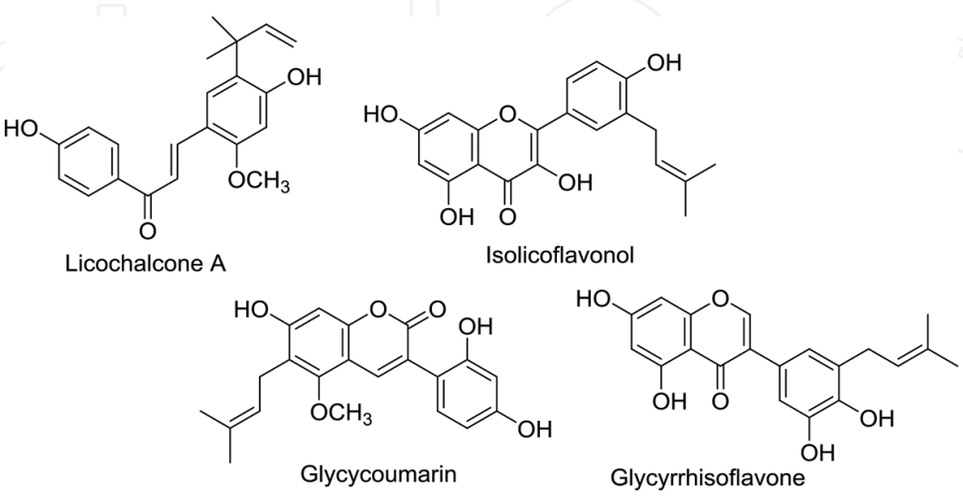

Figure 4. Licorice phenolics with anti-HIV effects.

2.3.3. Effects on drug-resistant bacteria

2.3.3.1. Polyphenols are effective against methicillin-resistant Staphylococcus aureus

Based on these studies, we pursued structural studies of licorice phenolics and also investigated the effectiveness of the licorice phenolics on drug-resistant bacteria. Surveillance under the Ministry of Health, Labour and Welfare within the Japanese government indicated that ca. 18,000 cases caused by methicillin-resistant *Staphylococcus aureus* (MRSA) in 2014 was reported for about 480 designated hospitals in Japan [33]. Since there are limited antibiotics and drugs (e.g., vancomycin and linezolid) available for the infectious diseases caused by MRSA, developing new candidates as remedies is essential. Indeed, hydrolyzable tannins such as tellimagrandin I and corilagin (in addition to an astringent constituent (-)-epicatechin gallate in green tea leaves) reportedly suppress the oxacillin resistance of MRSA strains. Therefore, we investigated licorice phenolics as candidates for new types of antibacterial drugs. Because acute toxicity is well understood for natural drug materials used in traditional medicine, low toxicity of their constituents is expected with some exceptions. Licorice has been widely used in traditional medicine, and its adverse effects (called pseudohyperaldosteronism) are ascribed to its main constituent glycyrrhizin. As such, its phenolic constituents could be candidates for developing novel remedies.

We examined the antibacterial effects of the phenolics isolated from licorice on four clinical isolates of MRSA (OM 481, OM505, OM 584, and OM 623) in addition to those of *Escherichia coli* and *Pseudomonas aeruginosa*. Although all of the examined phenolics did not show antibacterial effects on *E. coli* and *P. aeruginosa*, several compounds showed potent or moderate antibacterial effects on MRSA as shown below [15]. The compounds with a minimum inhibitory concentration (MIC) ≤ 32 μg/mL for the four MRSA strains are shown in **Table 2**. The following relationships were observed for both the structures and the antibacterial proper-

Subgroups	Compound names (MIC)	Substituents
Chalcones	Licochalcone A (16 μg/mL)	α,α-Dimethylallyl × 1, OH × 2
Isoflavones	8-(γ,γ-Dimethylallyl)-wighteone (8 μg/mL)	Prenyl × 2, OH × 3
	Gancaonin G (16 μg/mL)	Prenyl × 1, OH × 2
	Isowighteone (32 μg/mL)	Prenyl × 1, OH × 2
	Isoangustone A (16 μg/mL)	Prenyl × 2, OH × 4
Isoflavanones	3′-(γ,γ-Dimethylallyl)-kievitone (8 μg/mL)	Prenyl × 2, OH × 4
	Licoisoflavanone (32 μg/mL)	Dimethylpyran × 1, OH × 3
Isoflavans	Glabridin (16 μg/mL)	Dimethylpyran × 1, OH × 2
	Glyasperin C (16 μg/mL)	Prenyl × 1, OH × 3
	Glyasperin D (16 μg/mL)	Prenyl × 1, OH × 2
	Licoricidin (16 μg/mL)	Prenyl × 2, OH × 3
3-Arylcoumarins	Glycycoumarin (16 μg/mL)	Prenyl × 1, OH × 3
	Licoarylcoumarin (32 μg/mL)	α,α-Dimethylallyl × 1, OH × 3
2-Arylbenzofurans	Licocoumarone (16 μg/mL)	Prenyl × 1, OH × 3
Benzyl phenyl ketones	Glicophenone (32 μg/mL)	Prenyl × 1, OH × 4
	Licoriphenone (16–32 μg/mL)	Prenyl × 1, OH × 3

Table 2. Licorice phenolics effective on MRSA strains.

ties of these compounds. All of these compounds had two or more phenolic hydroxyl groups and at least one prenyl (γ,γ-dimethylallyl) or equivalent (α,α-dimethylallyl or dimethylpyran) group. Comparisons of the anti-MRSA properties of the chalcones examined indicated the importance of a prenyl (or equivalent) group such as licochalcone A (MIC 16 μg/mL) > echinatin (MIC 64 or 128 μg/mL), licochalcone B (MIC 128 μg/mL), liquiritigenin (MIC 128 μg/mL), and tetrahydroxymethoxychalcone (MIC >128 μg/mL). Indeed, isoflavones with two prenyl groups (8-(γ,γ-dimethylallyl)-wighteone [MIC 8 μg/mL] and isoangustone A [MIC 16 μg/mL]) showed more potent anti-MRSA effects than those with one prenyl group (isowighteone [MIC 32 μg/mL], glycyrrhisoflavone [MIC 32 or 64 μg/mL], and glisoflavone [MIC 64 μg/mL]). Together with 8-(γ,γ-dimethylallyl)-wighteone, an isoflavanone with two prenyl groups, 3′-(γ,γ-dimethylallyl)-kievitone had the most potent anti-MRSA effects (MIC 8 μg/mL) among the examined compounds (**Figure 5**). Similarly, isoflavans with prenyl or equivalent group(s) (i.e., glyasperins C and D, glabridin, and licoricidin) showed more potent anti-MRSA effects (MIC 16 μg/mL) than those without a prenyl group ((3*R*)-vestitol [MIC 128 μg/mL]). The role of the prenyl group was tied to its affinity for the bacterial cell membranes. On the other hand, methylation of phenolic hydroxyl (OH) groups on the same structural skeleton weakened the anti-MRSA properties: glycycoumarin (MIC 16 μg/mL) (1 × OMe) > glycyrin (MIC 128 μg/mL) (2 × OMe) > glycyrin permethyl ether (MIC >128 μg/mL) (4 × OMe). Comparing the MIC of glycycoumarin with that of the corresponding coumestan, glycyrol [MIC >128 μg/mL] suggested that skeleton flexibility is also a factor impacting the anti-MRSA effects.

We further examined the suppressive effects of licorice phenolics with relatively potent anti-MRSA effects on the oxacillin resistance of the MRSA strains [15]. We compared MICs of oxacillin on MRSA strains with and without phenolics at half the MIC concentration or lower. For example, the addition of 16 μg/mL isowighteone (MIC 32 μg/mL) decreased oxacillin MIC to 1/8–1/4 of those without the addition (e.g., from 512 to 64 μg/mL and from 64 to 16 μg/mL) for the four MRSA strains (**Figure 6**). Similarly, the addition of 8 μg/mL of isoangustone A (MIC 16 μg/mL) decreased oxacillin MICs to 1/4–1/2, and the addition of 16 μg/mL of glicophenone (MIC 32 μg/mL) decreased oxacillin MIC to 1/8–1/2. Most notably, the addition of 8 μg/mL of licoricidin caused a decrease of oxacillin MIC to lower than 0.5 μg/mL (lower than 1/1024–1/8). Even the addition of 4 μg/mL licoricidin decreased oxacillin MIC to 1/32–1/8 of those without the addition. Five of the other 6 phenolics, lico-

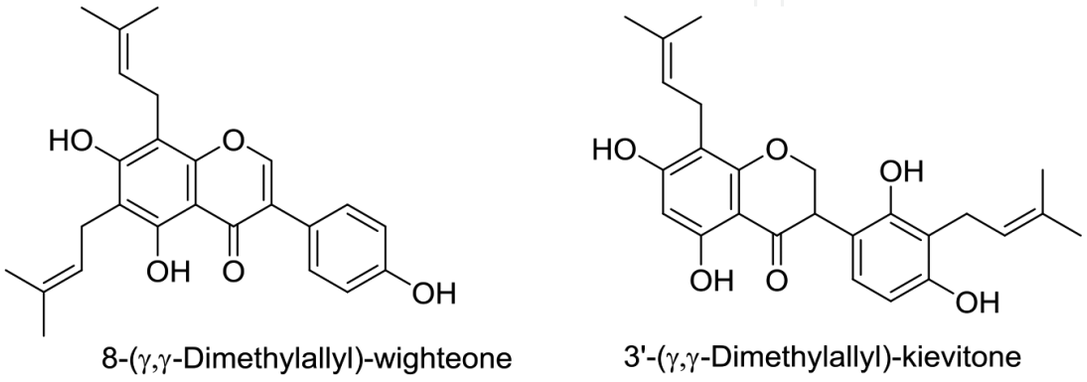

Figure 5. Licorice phenolics with the most potent antibacterial effect on MRSA.

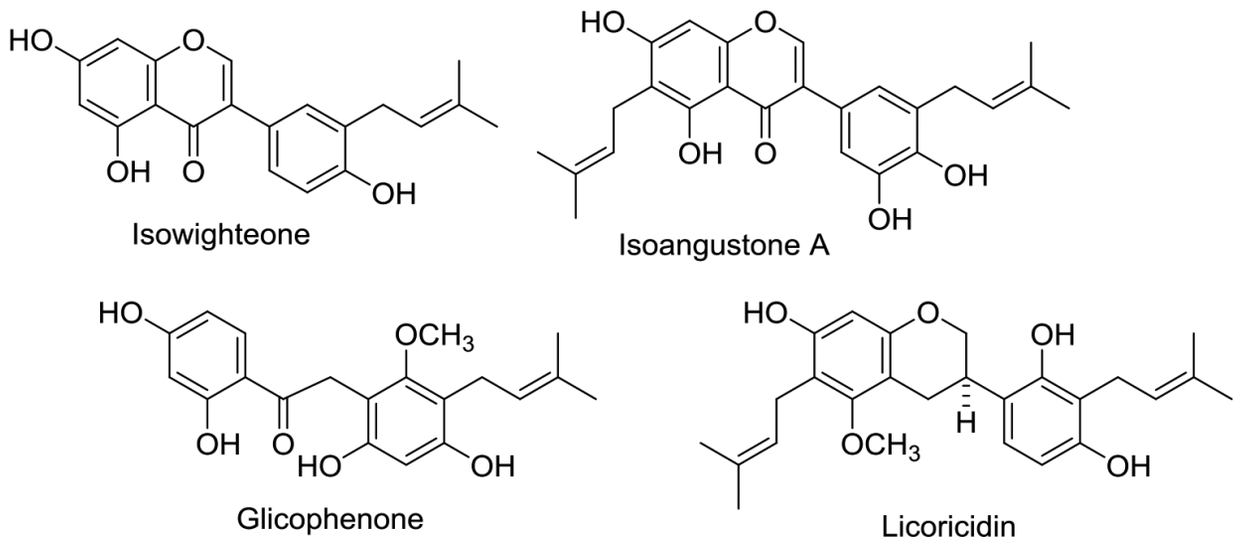

Figure 6. Licorice phenolics with suppressing effects on oxacillin resistance of MRSA.

chalcone A, licochalcone B, glicoricone, glisoflavone, and 3′-(γ,γ-dimethylallyl)-kievitone, also showed an analogous decreasing effect on at least two of the four MRSA strains. We also examined the effects of the combination of oxacillin (10 μg/mL) and licoricidin (8 μg/mL) on the bacterial growth of MRSA OM481, and the combination showed a bacteriostatic effect but not a bactericidal one. We also conducted a mechanistic study on the suppressive effects of the oxacillin resistance. The oxacillin resistance of MRSA OM481 has been attributed to the formation of a kind of protein-binding protein (PBP), PBP-2a (PBP-2′), instead of PBP-2. However, this formation in MRSA OM481 was not suppressed by the presence of licoricidin. Therefore, the suppression of the enzymatic function of PBP-2a or the binding to another PBP was attributed to the mechanism. On the other hand, the affinity of the lipophilic prenyl group to cell membranes was also supposed to be included in the mechanism, because all of the effective compounds have at least one prenyl (or equivalent) group.

Since the licorice phenolics with prenyl or equivalent substituents showed potent antibacterial effects on MRSA, we further investigated on the natural products with analogous structures contained in the fruits of *Psoralea corylifolia*, which have been known to have phenolic constituents with prenyl or related groups [34]. The following constituents of *P. corylifolia* showed MIC < 32 μg/mL for two MRSA strains, OM481 and OM584 (**Table 3**).

As shown above, the major constituent of the source material bakuchiol (meroterpene) together with isobavachalcone and corylifol B (chalcones) showed the most potent antibacterial effects among the constituents examined (**Figure 7**). We confirmed the importance of the presence of a prenyl or related lipophilic group in the molecules, suggesting that the participation of those groups is key within the bacterial membrane. Further mechanistic studies as shown by Refs. [35, 36] are expected.

2.3.3.2. Polyphenols are effective against vancomycin-resistant Enterococci

We further examined the effects of phenolic constituents of licorice on vancomycin-resistant *Enterococcus* (VRE) species. Most antibiotics are ineffective against VRE, and only a few drugs

Subgroups	Compound names (MIC)	Substituents
Flavones	Corylifol C (16 μg/mL)	Prenyl × 1, OH × 3
Flavanones	Bavachin (32 μg/mL)	Prenyl × 1, OH × 2
Isoflavones	Neobavaisoflavone (16 μg/mL)	Prenyl × 1, OH × 2
Chalcones	Corylifol B (8–16 μg/mL) Isobavachalcone (8 μg/mL)	Prenyl × 1, OH × 4 Prenyl × 1, OH × 3
Meroterpenes	Bakuchiol (8 μg/mL)	Ethenyldimethyloctadienyl × 1, OH × 1

Table 3. Phenolics from *Psoralea corylifolia* fruits effective on MRSA.

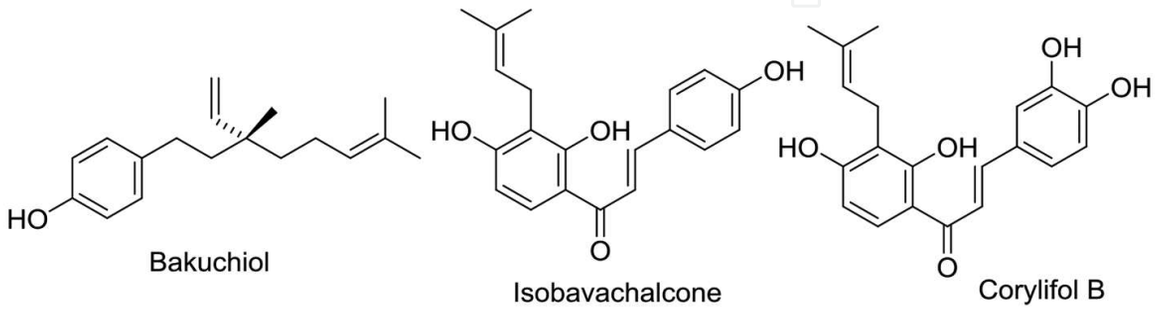

Figure 7. Phenolics with potent antibacterial effects on MRSA isolated from *Psoralea corylifolia* fruits.

Subgroups	Compounds (MIC)	Substituents
Isoflavones	8-(γ,γ-Dimethylallyl)-wighteone (8–16 μg/mL) Glycyrrhisoflavone (32 μg/mL) Isoangustone A (16 μg/mL) 7-*O*-Methylluteone (32 μg/mL) Semilicoisoflavone B (32–64 μg/mL)	Prenyl × 2, OH × 3 Prenyl × 1, OH × 4 Prenyl × 2, OH × 4 Prenyl × 1, OH × 3 Dimethylpyran × 1, OH × 3
Isoflavans	Glyasperin C (16 μg/mL) Glyasperin D (32–64 μg/mL) Licoricidin (8 μg/mL)	Prenyl × 1, OH × 3 Prenyl × 1, OH × 2 Prenyl × 2, OH × 3
Isoflavanones	3′-(γ,γ-Dimethylallyl)-kievitone (16 μg/mL) Glyasperin J (32 μg/mL)	Prenyl × 2, OH × 4 Dimethylpyran × 1, prenyl × 1, OH × 3
3-Arylcoumarins	Glycycoumarin (16 μg/mL) Glycyrin (16–32 μg/mL) Licoarylcoumarin (16 μg/mL)	Prenyl × 1, OH × 3 Prenyl × 1, OH × 2 α,α-Dimethylallyl × 1, OH × 3
Coumestans	Isoglycerol (32–64 μg/mL)	Dimethyldihydropyran × 1, OH × 1
Pterocarpans	Demethylhomopterocarpan (32 μg/mL)	OH × 1
2-Arylbenzofurans	Gancaonin I (8–16 μg/mL) Glycybenzofuran (32 μg/mL) Licocoumarone (32 μg/mL) 4′-*O*-Methylglycybenzofuran (32 μg/mL) Neoglycybenzofuran (16 μg/mL)	Prenyl × 1, OH × 2 Prenyl × 1, OH × 3 Prenyl × 1, OH × 3 Prenyl × 1, OH × 2 Prenyl × 1, OH × 3

Table 4. Licorice phenolics effective against VRE.

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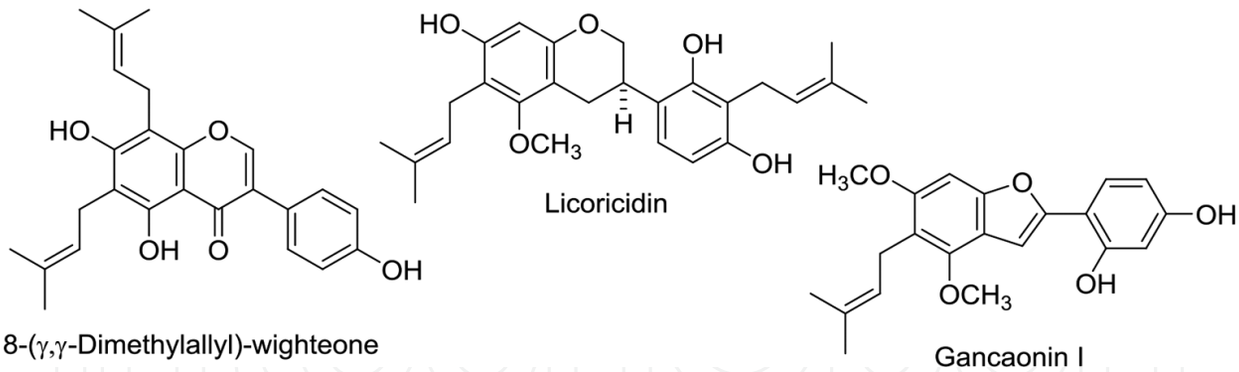

Figure 8. Licorice phenolics with potent antibacterial effects on VRE.

such as linezolid or daptomycin can be used for VRE. Approximately 60–120 of the infected cases have been reported annually in Japan [37]; thus, infection of VRE in hospitals has become an important issue. Therefore, we have also investigated the plant constituents that are effective against VRE [38, 39].

The following strains of two species of VRE, *E. faecium* FN-1 and *E. faecalis* NCTC12201, were used for this study on licorice constituents. Various types of licorice phenolics showed antibacterial effects on these two VRE species, as shown below. The compounds that showed antibacterial effects on VRE with an MIC ≤ 32 μg/mL were classified into skeletons of the compounds (**Table 4**) [18, 19]. The following relationships were observed for the structures and the antibacterial properties of these compounds. All of the compounds have prenyl or equivalent groups and at least one hydroxyl group. The compound that showed the most potent effects on VRE was licoricidin (MIC 8 μg/mL), an isoflavan that has two prenyl and three hydroxyl groups. Comparisons of the isoflavans identified the following order of the antibacterial effects: licoricidin (with two prenyl groups) > glyasperins C and D (with one prenyl group). Comparisons of the compounds with the same isoflavone skeleton revealed that 8-(γ,γ-dimethylallyl)-wighteone and isoangustone A (both had two prenyl groups) showed more potent antibacterial effects on VRE (MIC 8–16 μg/mL) than glycyrrhisoflavone and 7-*O*-methylluteone (MIC 32 μg/mL) (both had one prenyl group). Among the 3-arylcoumarins, the coumestans, and the 2-arylbenzofurans, the compound with the most potent antibacterial effects is gancaonin I (MIC 8–16 μg/mL), with two hydroxyl groups and a prenyl group (**Figure 8**).

The contribution of hydroxyl groups seems to be less important in the cases of VRE than in the case of MRSA. For example, isoglycyrol and demethylhomopterocarpan both contained one hydroxyl group and showed moderate effects with MIC 32–64 μg/mL. Even for glyasperin J trimethyl ether, which has no hydroxyl groups, an MIC of 64 μg/mL was observed for both of the VRE species. On the other hand, 6,8-diprenylorobol with two prenyl groups and four hydroxyl groups showed weak effects (MIC 128 μg/mL). Therefore, respective structural factors or some balance of lipophilicity and hydrophilicity may contribute to the antibacterial effects, and this should be further investigated.

2.4. High-performance liquid chromatographic analysis of licorice phenolics

HPLC analysis revealed the presence of characteristic constituents depending on the original plant species. The *Japanese Pharmacopoeia* indicates that licorice used as a medicinal material must be derived from the origins of *G. uralensis* and *G. glabra*. Our analytical investigation on licorice materials from various sources indicated that the HPLC profiles could be separated into the following three types depending on several major constituents [21].

Type A: Using HPLC analysis, the standard materials established as *G. uralensis* in China were found to contain three relevant compounds: glycycoumarin, licopyranocoumarin, and licocoumarone (**Figure 9**). Conversely, HPLC analysis of the standard materials of *G. glabra* and *G. inflata* did not indicate the presence of these three compounds. All of the materials obtained from Japanese markets contained glycycoumarin, licopyranocoumarin, and licocoumarone, and several materials from Chinese markets also showed analogous HPLC patterns.

Type B: Analogously, the standard materials from *G. glabra* identified in China contained glabridin and glabrene (**Figure 10**), whereas these two were not observed for the standard materials from *G. uralensis* and *G. inflata*. The materials from Russia and Afghanistan revealed these two constituents, which were absent in the Japanese and Chinese market products.

Type C: The standard materials from *G. inflata* included licochalcones A and B (**Figure 11**), which were also present in some of the materials from Chinese markets.

These results suggest that glycycoumarin, licopyranocoumarin, and licocoumarone could be used as markers for *G. uralensis* (Type A). At the same time, glabridin and glabrene could be used as markers for *G. glabra* (Type B), and licochalcones A and B could be used as markers for *G. inflata* (Type C). However, licochalcone B was later isolated from a Japanese market sample.

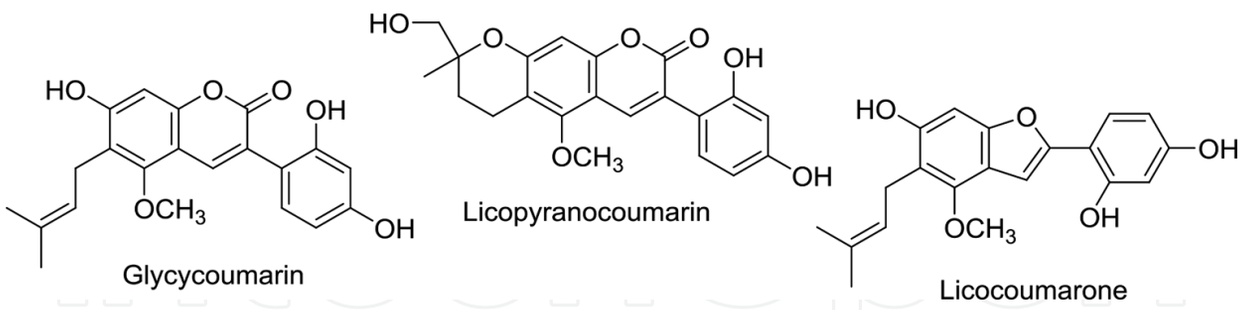

Figure 9. Characteristic phenolics observed in the extracts from *G. uralensis*.

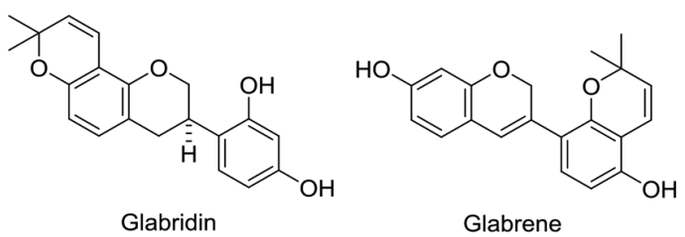

Figure 10. Characteristic phenolics observed in the extracts from *G. glabra*.

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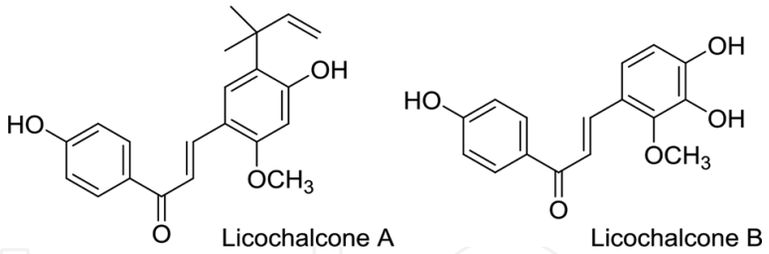

Figure 11. Characteristic phenolics observed in the extracts from *G. inflata*.

Furthermore, licoricidin, which was isolated from *G. uralensis* [15], has the same skeleton as glabridin. This finding suggests that glabridin might be a common constituent of *G. uralensis* and *G. glabra*, an assertion that is further strengthened by the fact that glabridin was recently found from *G. uralensis* [40]. Therefore, reinvestigation of marker compounds may be required, although glabrene and licochalcone A can be considered markers for *G. glabra* and *G. inflata*, respectively.

We performed HPLC analysis for the evaluation of crude drug materials to ascertain their pharmacological effects. The simultaneous HPLC analysis of eight major constituents of an extract from a material of a Japanese market was performed for evaluation as an anti-VRE material [19]. Using HPLC instruments combined with a photodiode-array detector (DAD) (LC-UV) or mass spectrometer (LC-MS) [19] would also effectively characterize such crude drug materials. Quantitative data and comparisons of the chromatographic patterns of representative licorice extracts, including unidentified HPLC peaks, are contributable to the evaluation of the materials. In addition, thin-layer chromatography (TLC) is a very useful method for visualizing phenolic constituents in plant extracts without special instruments [41], and development of high-performance (HP)TLC technique resulting in a better resolution of the constituent spots contributes largely in the analysis of plant constituents [42]. High-performance size-exclusion chromatography can be applied for estimating molecular sizes or molecular weight distribution of tannins [43] and also for estimating sizes of supermolecular complexes formed from polyphenols and proteins [26]. Gel electrophoresis is applicable for the analyses of polyphenol-protein complexes [44], too.

3. Conclusions

Licorice extracts contain various types of flavonoids and related compounds. In addition to the protein-binding properties and antioxidant effects, we examined their antiviral and antibacterial properties. The findings, especially those found in the studies of antibacterial phenolics in licorice using MRSA and VRE, emphasize the importance of lipophilic prenyl groups together with phenolic hydroxyl groups, in addition to the flexibility of their structural skeletons. Additional studies on these plant constituents are currently in progress [45]. Because naturally occurring polyphenols have structural limitations based on the biogenetic capability of plants, further studies with the aid of synthetic chemistry are expected for clarifying quantitative structure-activity relationship concerning their pharmacological effects and for optimizing candidates of new drugs.

4. Notes

The author (TH) regrets that the following errors were found: (1) The concentration 1 μg/mL of oxacillin in the figure legend on the effects of the combination of oxacillin and licoricidin from **Figure 2** in Ref. [15] should read 10 μg/mL as shown in the text of Ref. [15]; (2) the methoxyl and the hydroxyl groups in the structure of glycyrol in Refs. [18, 19] should be at C1 and C3, respectively, as shown in Ref. [34], and the structure of glycycoumarin in the Refs. [18] and [19] should be fixed as shown in Refs. [9, 15]; (3) the subgroup name 2-aryl-3-methylbenzofuran for gancaonin I in Ref. [19] is incorrect. Because gancaonin I does not have a methyl group on C3, it is classified in a subgroup of 2-arylbenzofuran as shown in **Table 4** in this chapter; (4) the name "reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)" in Ref. [30] is an error and should read "NADH."

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