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Valorisation of liquorice (*Glycyrrhiza*) roots: antimicrobial activity and cytotoxicity of prenylated (iso)flavonoids and chalcones from liquorice spent (*G. glabra*, *G. inflata*, and *G. uralensis*)†

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Prenylated phenolics are antimicrobials found in liquorice (*Glycyrrhiza* spp.). Liquorice spent is a by-product rich in prenylated phenolics obtained after water extraction of roots, and is currently not valorised. We analysed the prenylated phenolics composition of spent extracts from *Glycyrrhiza glabra*, *G. inflata*, and *G. uralensis*, their antimicrobial activity, cytotoxicity, and effects on Caco-2 cell viability. *G. glabra*, *G. inflata*, and *G. uralensis* spent extracts showed distinct phytochemical profiles. Antibacterial activity (*Lactobacillus buchneri*, *Streptococcus mutans*, and *Staphylococcus aureus*) of *G. uralensis* and *G. inflata* (MICs 25–250 µg mL⁻¹) was higher than of *G. glabra* (MICs 75–1000 µg mL⁻¹). Marker compounds glabridin, licochalcone A, and glycy coumarin were equally potent (MICs 12.5–25 µg mL⁻¹). *G. inflata* and *G. uralensis* showed cytotoxicity at 500 µg mL⁻¹, whereas *G. glabra* was not toxic up to 1000 µg mL⁻¹, but showed reduced viability between 50–500 µg mL⁻¹. Linking antibacterial activity of the liquorice spent extracts with cell viability showed that MICs against *S. aureus* coincide with concentrations where cell viability was not reduced, whereas for the other bacteria and yeasts MICs concurred at concentrations where cell viability was reduced. In this study we show that liquorice spent is a by-product rich in antibacterial prenylated phenolics that offers interesting opportunities for e.g. control of microorganisms and the discovery of novel plant-derived antimicrobials.

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1. Introduction

The *Glycyrrhiza* genus comprises different species, with *G. glabra*, *G. inflata*, and *G. uralensis* being the best known commercially used. Water extracts of liquorice roots are *i.a.* used as a flavouring agent, but have also a longstanding use in traditional herbal medicine, used to treat *i.a.* cough, inflammation, and liver damage.^{1,2} As of 2012, the European Medicines Agency (EMA) has recommended the use of liquorice roots for their pharmacological properties.³ The world's demand for liquorice extract is increasing, with a global liquorice extract market value of \$1.9 billion in 2019, which is anticipated to increase towards \$2.9 billion by 2029.^{4,5} Glycyrrhizin

(a triterpenoid saponin) is the main compound responsible for the liquorice flavour and sweetness.⁶ Besides glycyrrhizin, liquorice roots are rich in prenylated (iso)flavonoids and chalcones (collectively referred to as prenylated phenolics, for included subclasses see Fig. A1, ESI†). These compounds have shown to possess potent antimicrobial activity.^{7,8} Natural antimicrobials are in high demand as alternatives for existing antimicrobials that face increased resistance and often become part of the human food chain.⁹ In general, there is an increasing consumer interest for minimal processed and "natural" products.¹⁰ Specialised ('secondary') metabolites from plants, either as pure compounds or as extracts, may provide novel antimicrobials.¹¹ Under (a)biotic stress, prenylated phenolics are produced by plants from the *Fabaceae* (Leguminosae) family, including liquorice roots (*Glycyrrhiza* spp.).¹² Recently, we showed that an ethyl acetate (EtOAc) extract of *G. glabra* roots is rich in prenylated phenolics, with prenylated isoflavans being the most abundant.¹³ Additionally, it was shown that methanolic root extracts of *G. glabra*, *G. inflata*, and *G. uralensis* each have a distinct prenylated phenolic profile:

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G. glabra is rich in prenylated isoflavans, *G. inflata* in prenylated chalcones, and *G. uralensis* in prenylated isoflavans.^{14–17} Furthermore, these studies revealed considerable interspecies chemical variation, by discovering 27 species-specific compounds, the most abundant species-specific compounds were glabridin (glab), licochalcone A (licoA), and glycycoumarin (glycy) for *G. glabra*, *G. inflata*, and *G. uralensis*, respectively (Fig. 1).¹⁴ Previous research has established antibacterial activity of the aforementioned compounds, with minimum inhibitory concentrations (MICs) of 49 μM (16 $\mu\text{g mL}^{-1}$) for glab, 77 μM (26 $\mu\text{g mL}^{-1}$) for licoA, and 44 μM (16 $\mu\text{g mL}^{-1}$) for glycy against methicillin-resistant *Staphylococcus aureus* (MRSA).¹⁸ Natural compounds with a MIC $\leq 25 \mu\text{g mL}^{-1}$ are considered potent antibacterials.¹⁹

In general, prenylation of phenolics increases their antimicrobial activity, as was demonstrated in a previous study from our group, reporting MICs of 29 μM (10 $\mu\text{g mL}^{-1}$) for 6-prenylenstein (single prenylated) and 15 μM (6.3 $\mu\text{g mL}^{-1}$) for 6,8-diprenylenstein (double prenylated), against the food pathogen *Listeria monocytogenes* EGD-e.²⁰ Reported MIC values of the non-prenylated counterpart (genistein) range between 237–3704 μM (64–1000 $\mu\text{g mL}^{-1}$) against a variety of Gram-positive and Gram-negative bacteria.^{21,22}

It has been proposed that prenylation leads to an increased antimicrobial activity by enhancing the hydrophobicity of the molecule, which leads to more interaction with- and/or disruption of bacterial membranes.²³ As this might also hold for human cell membranes, concern regarding their safety for consumption is raised. Data from several studies show that prenylated phenolics induce cytotoxicity and reduce cell viability in different human cancer cell lines (with IC₅₀ between 10–85 μM for prenylated chalcones and flavanones in HT-29 colon cells).^{24–27} However, possible cytotoxicity and potential effects on cell viability from complex liquorice spent extracts rich in prenylated phenolics has not been investigated before.

To date, liquorice roots are commercially used for *e.g.* glycyrrhizin extraction, leaving the spent as potential, thus far untapped source of bioactive compounds. With an average global annual liquorice water extract content of 58 kiloton (kt),²⁸ it is expected that 232 kt spent is produced yearly (assuming a 25% (*w/w*) water extract yield), containing around 1.2 kt prenylated phenolics.¹³ Therefore, the by-product of liquorice roots after water extraction, also referred to as spent,

was studied as a source of potent and non-toxic antimicrobial prenylated phenolics. To the best of our knowledge, the use of liquorice spent as source of antimicrobial prenylated phenolics has not been investigated before. As compounds glab, licoA, and glycy have shown potent antibacterial activity, valorisation of liquorice spent seems to offer interesting opportunities for *e.g.* control of microorganisms and the discovery of novel plant-derived antimicrobials. Therefore, the aim of this study is to get a comprehensive overview of the phytochemical composition, antimicrobial potential, and general toxicity of liquorice spent extracts and species-specific marker compounds glab, licoA, and glycy. For this, the phytochemical composition of *G. glabra*, *G. inflata*, and *G. uralensis* spent extracts was studied using reversed phase ultra-high pressure liquid chromatography (RP-UHPLC) coupled to photodiode array (PDA), ion trap mass spectrometry (IT-MSⁿ) and Orbitrap-MS (FT-MSⁿ). Antimicrobial activity of spent extracts and marker compounds was evaluated against food spoilers (preservation) *Lactobacillus buchneri*, *Zygosaccharomyces parabailii*, and *Yarrowia lipolytica*, oral pathogens (dental hygiene) *Streptococcus mutans* and *Staphylococcus aureus*, and the gastrointestinal (colonic health and fecal contamination) *Escherichia coli*. Last, potential effects on cytotoxicity (LDH) and cell viability (WST-1) of the spent extracts and marker compounds were evaluated in human colorectal adenocarcinoma cells (Caco-2 cells), a cell line that is widely used as *in vitro* screening model for intestinal barrier function and also in cytotoxicity studies.²⁹

2. Materials and methods

2.1. Materials

Liquorice roots, *Glycyrrhiza glabra* (Iran), *G. inflata* (China), and *G. uralensis* (China) were provided by Ruitenberg BasIQs BV (Twello, The Netherlands). Glabridin (glab) ($\geq 97.0\%$) was purchased from Wako (Osaka, Japan); licochalcone A (licoA) ($\geq 96.0\%$), *tert*-butanol $\geq 98\%$ (*w/w*), and Triton X-100 were from Sigma-Aldrich (St Louis, MO, USA); glycycoumarin (glycy) ($\geq 98.0\%$) from Chemfaces (Wuhan, China; confirmed by RP-UHPLC-PDA-FT-MSⁿ); ULC-MS grade ethyl acetate (EtOAc), methanol (MeOH), acetonitrile (ACN), ACN containing 0.1% (*v/v*) formic acid (FA), water containing 0.1% (*v/v*) FA were purchased from Biosolve (Valkenswaard, The Netherlands); dimethyl sulfoxide (DMSO) was purchased from Merck Millipore (Billerica, MA, USA). Water (MQ) for other purposes than UHPLC was prepared using a Milli-Q water purification system (Merck Millipore). Growth media, Bacto brain heart infusion (BHI) was purchased from BD (Franklin Lakes, NJ, USA); tryptone soya broth (TSB) and agar (TSA) bacteriological and DeMan-Rogosa-Sharpe (MRS) broth were obtained from Oxoid Ltd (Basingstoke, UK), yeast extract peptone dextrose broth (YPD) from Brunschwig Chemie (Amsterdam, The Netherlands), and peptone physiological salt solution (PPS) from Tritium Microbiologie (Eindhoven, The Netherlands). Phosphate buffered saline (PBS) and Dulbecco's modified Eagle's medium

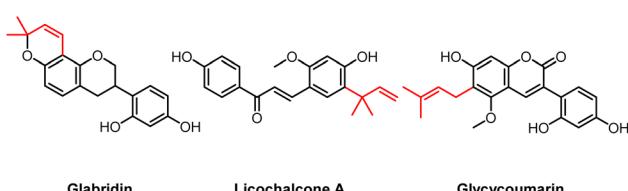


Fig. 1 Molecular structures of the main species-specific compounds glabridin (glab) (ring prenylated isoflavan from *G. glabra*), licochalcone A (licoA) (chain prenylated chalcone from *G. inflata*), and glycycoumarin (glycy) (chain prenylated 3-arylcoumarin from *G. uralensis*). The prenyl group is indicated in red.



(DMEM) were purchased from Thermo Fisher Scientific Gibco (Waltham, Massachusetts, USA) and supplemented with glucose (4.5 g L⁻¹), HEPES buffer solution (0.58 g L⁻¹), L-glutamine (0.58 g L⁻¹), 10% (v/v) heat inactivated fetal calf serum (FCS), and 1% (v/v) penicillin/streptomycin (P/S).

2.2. Microorganisms and mammalian cell line

Lactobacillus buchneri ATCC 4005, *L. buchneri* L4, *Zygosaccharomyces parabailli* ATCC 60483, and *Z. parabailli* UL 3699 were provided by Unilever (Unilever Foods Innovation Centre – Hive, Wageningen, The Netherlands); *Streptococcus mutans* (ATCC 27175) was purchased at DSMZ (Braunschweig, Germany); *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (K12) were provided by the Laboratory of Food Microbiology (Wageningen University, Wageningen, The Netherlands); *E. coli* field isolates O54:H21 and O88:H8 were provided by Delacon Biotechnik GmbH (Engerwitzdorf, Austria); *Yarrowia lipolytica* food isolate was provided by Ruitenberg BasiQs BV (Twello, The Netherlands). Human colon adenocarcinoma cell line (Caco-2) was obtained from American Type Culture Collection (ATCC; Manassas, Virginia, US).

2.3. Sample preparation and extraction

Liquorice roots were freeze-dried prior to milling in a Retsch SM 2000 (Retsch, Haan, Germany), and subsequently sieved (pore size 250 µm, Retsch) to yield root powder. The resulting powder was extracted with MQ by ultrasound-assisted extraction (1 : 25 [w:w]) in 2 consecutive cycles of 60 min per cycle at 65 °C with extra shaking every 15 min, and the supernatants (after centrifugation for 10 min at 4696g at 4 °C) of both cycles were combined to yield the water extracts. Water extracts were freeze-dried (Christ Alpha 1-4 LD, Osterode am Harz, Germany) prior to compositional analysis by UHPLC-MS. Obtained pellet was referred to as spent and freeze-dried (Christ) prior to extraction with EtOAc. Subsequently, liquorice spent was extracted with EtOAc by ultrasound-assisted extraction (1 : 25 [w:w]) in 2 consecutive cycles of 60 min per cycle at 35 °C with extra shaking every 15 min. EtOAc extracts were centrifuged for 10 min at 4696g at room temperature (RT) and combined to yield spent extract. EtOAc in the spent extract was removed under reduced pressure and dried extract was resolubilized in *tert*-butanol and freeze-dried. Prior to UHPLC-MS analysis, the dried spent extract and standards were resolubilized in DMSO to a final concentration of 1 mg mL⁻¹ and 100 µg mL⁻¹ for ESI-IT-MSⁿ analysis, or 50 µg mL⁻¹ and 1 µg mL⁻¹ for ESI-FT-MSⁿ analysis, respectively; dried water extract was resolubilized in MQ to a final concentration of 5 mg mL⁻¹ and analysed by ESI-IT-MSⁿ. Prior to injection into the UHPLC-MS, all extracts and standards were centrifuged at 15 000g for 5 min at RT.

2.4. Reversed phase liquid chromatography (RP-UHPLC-PDA)

Water extracts, spent extracts, and standards were separated on a Thermo Vanquish UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, degasser, autosampler and photodiode array (PDA) detector. Identical column,

eluents, and gradient elution program were used as reported by van Dinteren *et al.*¹³

2.5. Electrospray ionisation ion trap mass spectrometry (ESI-IT-MSⁿ)

Mass spectrometric data were acquired using a LTQ Pro linear ion trap mass spectrometer (Thermo Scientific) equipped with a heated ESI probe coupled in-line to the Vanquish UHPLC system, as described elsewhere.¹³ For the water extracts, data were collected in negative ionisation (NI) and positive ionisation (PI) mode between *m/z* 200–2000. Besides, data-dependent MSⁿ analyses by collision-induced dissociation with a normalized collision energy (NCE) of 35%, also NCE of 20% was used. MSⁿ fragmentation was performed on the most intense product ion in the MSⁿ⁻¹ spectrum, as well as on characteristic fragments related to prenylation in PI mode ([M + H - C₃H₆]⁺ and [M + H - C₄H₈]⁺).

2.6. Electrospray ionisation hybrid quadrupole orbitrap mass spectrometry (ESI-FT-MSⁿ)

Accurate mass data were acquired using a Thermo Q Exactive Focus hybrid quadrupole-Orbitrap Fourier transform mass spectrometer (FT-MS) (Thermo Scientific) equipped with a heated ESI probe, as described elsewhere.¹³

2.7. Identification of prenylated phenolics in water and spent extracts

Prenylated phenolics were tentatively identified based on our recently developed prenylation decision guideline,¹³ based on UV-Vis, and MS spectral data, obtained by Xcalibur 4.1. A summary of different UV-Vis absorption spectra and *retro*-Diels-Alder (RDA) fragmentation patterns is provided in Table B1 (ESI†).

2.8. Quantification of prenylated phenolics in water and spent extracts

Quantification of phenolics was based on their UV absorbance at 280 nm, applying a cut-off rule that the peak should account for at least 0.75% of the total UV area at 280 nm. For this, six-point (0.1–100 µg mL⁻¹) calibration curves based on external standards of glab, licoA, and glycy were used (*R*² = 0.999). UV peaks were integrated using the AVALON integration algorithm with the auto-calc function (Xcalibur 4.1). Subsequently, content of each compound was corrected for the differences in molar extinction coefficients between the standard and the compounds of interest, using a derivative of Lambert-Beer's law (eqn (1)).

$$C_i = \frac{\text{Area}_{280}}{k_{\text{standard}} \times ((\epsilon_i/M_{w,i})/(\epsilon_{\text{standard}}/M_{w,\text{standard}}))} \quad (1)$$

In which *C_i* is the concentration of the compound that is quantified, *Area₂₈₀* is the integrated area of the UV peak at 280 nm, *k_{standard}* is the response factor or slope of the standard (glab, licoA, or glycy), *ε* (AU M⁻¹ cm⁻¹) is the molar extinction coefficient, and *M_w* is the molecular weight (g mol⁻¹). Because



the ϵ -values of most tentatively identified compounds were unavailable, for each (iso)flavonoid subclass a representative compound was chosen and its ϵ reported in literature was used (corrected for the wavelength using $\epsilon_i = \epsilon_r \times \frac{A_i}{A_r}$, in which A is the UV absorbance, i is the wavelength at which the compound is quantified, and r is the wavelength reported in literature, Table C1, ESI†).

To evaluate the effect of the sample matrix on the performance of the quantification method, EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* spent were spiked with glab, licoA, or glycy at 25 and 50 $\mu\text{g mL}^{-1}$. Recovery was calculated with eqn (2).

$$R(\%) = \frac{x_{\text{spiked}} - x_{\text{unspiked}}}{x_{\text{ref}}} \times 100 \quad (2)$$

In which x_{spiked} is the measured concentration of the analyte in the spiked experiment, x_{unspiked} is the measured concentration of the analyte in the unspiked experiment, and x_{ref} is the true concentration which was spiked to the extract. Recovery of glab ranged between 97–102%, of licoA between 104–120%, and of glycy between 120–128%. Obtained recoveries were acceptable, as a good recovery should be between 80–120%.³⁰

2.9. Antimicrobial activity assay by the broth microdilution assay

Bacteria (*L. buchneri* ATCC 4005, *L. buchneri* L4, *S. mutans* ATCC 27175, *S. aureus* ATCC 25923, *E. coli* K12, *E. coli* O54:H21, *E. coli* O88:H8), and yeasts (*Y. lipolytica* food isolate, *Z. parabailii* ATCC 60483, *Z. parabailii* UL 3699) were streaked from a glycerol stock to their optimal growth medium agar plate and incubated for 24–72 h for bacteria and 72 h for yeasts at 25–37 °C. One colony was transferred to corresponding broth and further incubated for 18–24 h at the microorganisms' optimal growth temperature. Overnight cultures were diluted 50–10 000 \times to obtain an initial inoculum concentration of $\sim 5.0 \log_{10}$ CFU mL^{-1} . An overview of the optimal growth media, incubation temperatures, incubation times, and final initial inoculums of each microorganism is shown in Table D1 (ESI†).

Stock solutions of the liquorice spent extracts (100 mg mL^{-1}) and species-specific marker compounds glab, licoA, and glycy (20 mg mL^{-1}) were prepared in DMSO. Stock solutions of control compounds chlorhexidine, ampicillin, and carvacrol were prepared in DMSO at 20, 10, and 10 mg mL^{-1} , respectively. Potassium sorbate stock was prepared at 250 mg mL^{-1} in MQ. Equal volumes (100 μL) of bacterium or yeast inoculum and a series of 2-fold dilutions of liquorice spent extracts (25–2000 $\mu\text{g mL}^{-1}$, microorganism-dependent) and marker compounds (3.125–200 $\mu\text{g mL}^{-1}$, microorganism-dependent) in growth medium were mixed into a 96-wells plate (maximum concentration of DMSO concentration was 2.0% (v/v), which did not affect bacterial/fungal growth, Table M1, ESI†). The 96-wells plate was incubated in a Spectramax ID3 (Molecular Devices, Sunnyvale, CA, USA) or in a Tecan Infinite 200Pro (Tecan Group Ltd, Zürich, Swiss) for 24–72 h. Microbial incu-

bation was set at the microorganisms' optimal growth temperature and time (Table D1†) with double orbital and high intensity shaking before each measurement and medium intensity shaking in-between each measurement for 5 seconds. Tecan incubation was set at the microorganisms' optimal growth temperature and time (Table D1†) applying orbital shaking with an amplitude of 3 mm and frequency of 218.3 rpm, before each measurement for 5 seconds.

Growth was determined by measuring the optical density (OD) at 600 nm every 10 min. Time to detection (TTD) was defined as the time to reach a change in OD of 0.05 units.³¹ When no reliable TTD could be obtained, growth delay ratio was determined; growth delay ratio was defined as the ratio between intercept B in the presence of an antimicrobial compound and intercept A in the absence of antimicrobial compound (negative control), as is shown in Fig. G1.† Positive controls (chlorhexidine for *S. mutans* and *S. aureus*, carvacrol and ampicillin for *E. coli*, potassium sorbate for *L. buchneri*, *Z. parabailii*, and *Y. lipolytica*), negative controls (growth medium suspension of bacteria/yeasts with 2.0% (v/v) DMSO), and blanks (extracts and growth medium without bacteria/yeasts) were considered for optical comparison and sterility control. Liquorice spent extracts and species-specific markers were tested in three biological replicates, in duplicate or triplicate.

When ΔOD was less than 0.05 (or when flat lines after the incubation period were obtained), viable cell count was performed. In brief, 100 μL from each well was decimaly diluted in PPS and spread onto optimal growth medium agar plate. Plates were incubated at the optimum growth temperature and time (Table D1†) after which colonies were counted.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of extract or compound that resulted in a cell count equal or lower than that of the initial inoculum. The minimum bactericidal or fungicidal concentration (MBC or MFC) was determined as the lowest concentration of extract or compound that resulted in >99% bacteria or yeast inactivation from the initial inoculum. Minimum inhibitory prenylated phenolics concentrations (MIPPC) and minimum bactericidal/fungicidal prenylated phenolics concentrations (MBPPC/MFPPC) were determined, which was the MIC or MBC corrected for the content of prenylated phenolics. For glab, licoA, and glycy the MIC/MBC/MFC was the same as the MIPPC/MBPPC/MFPPC.

To determine the role of molecular efflux pumps in the resistance of *E. coli* towards the liquorice spent extracts, the efflux pump inhibitor phenylalanine-arginine β -naphthylamide dihydrochloride (Pa β N) was added with the liquorice spent extracts during the broth microdilution assay. Pa β N inhibits AcrAB-TolC, the most prominent efflux pump in *E. coli*.^{32–34} Pa β N was added in growth medium at 25 $\mu\text{g mL}^{-1}$ (which did not affect bacterial growth, data not shown).

Natural compounds with a MIC $\leq 15 \mu\text{g mL}^{-1}$ are considered highly active antimicrobial compounds, with a MIC between 15–25 $\mu\text{g mL}^{-1}$ they are classified as active antimicrobial compounds, compounds with MICs between



25–100 $\mu\text{g mL}^{-1}$ are considered moderately active antimicrobials, and those with MICs $>100 \mu\text{g mL}^{-1}$ are considered as inactive.¹⁹

2.10. Cell culture

Caco-2 cells were cultured in Dulbecco's Modified Eagle's supplemented with glucose (4.5 g L^{-1}), HEPES buffer solution (0.58 g L^{-1}), L-glutamine (0.58 g L^{-1}), 10% (v/v) heat-inactivated fetal calf serum, and 1% (v/v) penicillin and streptomycin, in a humidified atmosphere of 5% CO_2 at 37 °C until 80–90% confluence. Cells between passages 14 and 33 were used. To assess cell cytotoxicity (section 2.11) and cell viability (section 2.12), cells were seeded into 96-wells plates at a density of 15 000 cells per cm^2 (or 4800 cells per well). Cells were differentiated for 21 days and medium was changed every 48 h.

2.11. Cell cytotoxicity

Cytotoxic effects of EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* (12.5–1000 $\mu\text{g mL}^{-1}$) and marker compounds glab, licoA, and glycy (3.125–100 $\mu\text{g mL}^{-1}$) on Caco-2 cells were assessed after 4 h of incubation, by measuring leakage of intracellular lactate dehydrogenase (LDH) in supernatant and analysis using an LDH cytotoxicity detection kit (Roche Applied Science, Almere, The Netherlands), according to manufacturer's instructions. Liquorice spent extracts and marker compounds were dissolved in DMSO at 100 mg mL^{-1} and 10 mg mL^{-1} , respectively. Highest concentration of DMSO in measurements was 1% (v/v), which did not affect cell cytotoxicity (Fig. N1, ESI†). LDH activity in the supernatant was expressed as percentage of the maximum releasable LDH in cells (Caco-2 cells treated with 1% (v/v) Triton X-100) and calculated with eqn (3).HH

$$\text{Cytotoxicity (\%)} = \frac{\text{exp. value} - \text{low control}}{\text{high control} - \text{low control}} \times 100 \quad (3)$$

here, exp. value is UV absorbance at 492 nm (Multiskan Ascent, Thermo Fisher Scientific), low control is the spontaneous LDH release in untreated cells (Caco-2 cells in culture medium), and high control is the maximum releasable LDH in cells (Caco-2 cells treated with 1% (v/v) Triton X-100). Liquorice extracts and standards were considered cytotoxic when $>20\%$ cytotoxicity was observed.

To evaluate the stability of glab, licoA, and glycy in DMEM, 50 $\mu\text{g mL}^{-1}$ of each standard was incubated for 4 h at 37 °C, after which glab, licoA, and glycy were quantified based on UV absorbance at 280 nm (section 2.8). For this, six-point (1–100 $\mu\text{g mL}^{-1}$) calibration curves of external standards of glab, licoA, and glycy were used ($R^2 \geq 0.999$). Glab, licoA, and glycy were stable in DMEM, with recovery of 102 ± 6.3%, 101 ± 14%, and 106 ± 8.8%, respectively.

2.12. Cell viability

Effects on cell viability in Caco-2 cells by EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* (12.5–1000 $\mu\text{g mL}^{-1}$) and marker compounds glab, licoA, and glycy (3.125–100 μg

mL^{-1}) were assessed after incubation of 4 h, by measuring cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases, and analysed using a WST-1 cell viability kit (PromoKine, Heidelberg, Germany), according to manufacturer's instructions. Cell viability was expressed as percentage of the control cells (Caco-2 cells in culture medium) and calculated with eqn (4).

$$\text{Viability (\%)} = \frac{\text{exp. value}}{\text{low control}} \times 100 \quad (4)$$

here, exp. value is UV absorbance at 450 nm (Multiskan Ascent, Thermo Fisher Scientific) and low control is spontaneous cleavage of WST-1 to formazan by mitochondrial dehydrogenases in untreated cells.

2.13. Statistical data analysis

Cell cytotoxicity and viability data were analysed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics v.25 software (SPSS Inc., Chicago, IL, USA). Normality was confirmed by checking Q-Q plots. When equal variances were assumed (by Levene's test), significant differences ($p \leq 0.05$) were compared using Dunnett's *post hoc* comparisons. When equal variances were not assumed, significant differences ($p \leq 0.05$) were compared using Dunnett's T3 *post hoc* comparisons.

3. Results and discussion

3.1. Prenylated phenolics composition of *Glycyrrhiza glabra*, *G. inflata*, and *G. uralensis* spent extracts

Representative chromatographic profiles of the EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* are shown in Fig. 2. In total, 41 peaks for *G. glabra*, 36 peaks for *G. inflata*, and 32 peaks for *G. uralensis* were selected (corresponding to 74%, 81%, and 75% of the total UV area at 280 nm, respectively). Using our recently developed prenylation decision guideline¹³ and the established fragmentation behaviour of different classes of phenolics (Table B1, ESI†), aforementioned peaks were annotated. Annotations are listed in Table E1 (IT-MSⁿ) and Table F1 (FT-MSⁿ) (ESI†).

The total content of annotated compounds in the EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* was 105 ± 13 (10.5 ± 0.13% w/w), 187 ± 10 (18.7 ± 0.10% w/w), and 196 ± 20 (19.6 ± 0.20% w/w) mg per g DW extract, of which 96, 171, and 155 mg per g DW extract were prenylated phenolics. Estimated content of each individual compound is shown in Table H1 (ESI†). An overview of the tentative identification (in positive ionisation mode IT-MSⁿ and HR-MS) and content of the top four major prenylated phenolics (from Table H1†) per liquorice species, including species-specific markers is shown in Table 1. In a recent study by Song and co-workers, phenolic content in methanolic root extracts of *G. glabra*, *G. inflata*, and *G. uralensis* was 65%.¹⁴ Methanolic extraction leads to extraction of a wider range of non-prenylated non-flavonoid phenolics, also resulting in a higher phenolics content compared to our extraction (content ranging between 10.5% and 19.6%



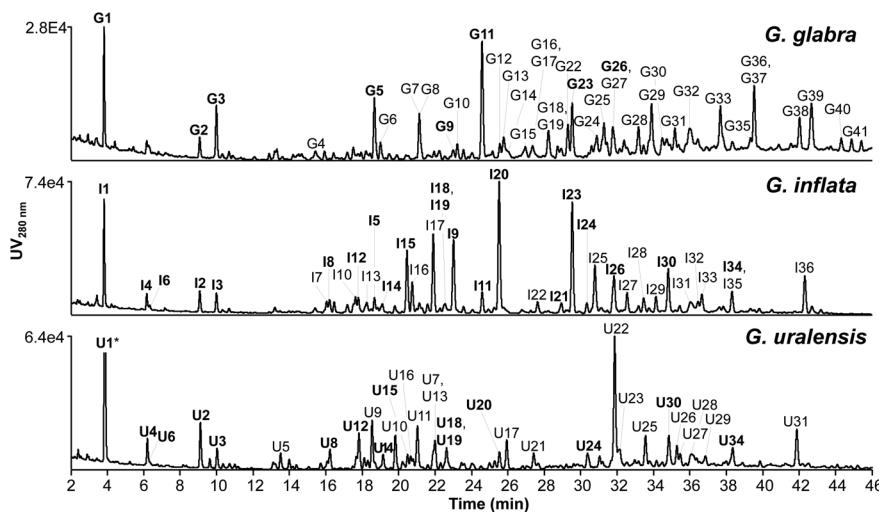


Fig. 2 RP-UHPLC-PDA chromatograms (at 280 nm) of EtOAc spent extract of *Glycyrrhiza glabra*, *G. inflata*, and *G. uralensis*. Peaks labelled with a number indicate (tentatively) identified phenolic compounds (Table E1 and F1, ESI†). Bold similar numbers indicate the same compounds identified in at least two *Glycyrrhiza* species (G = *G. glabra*, I = *G. inflata*, and U = *G. uralensis*). * Peak U1 was cut at an intensity of 2.44×10^5 .

between the different liquorice species). Non-prenylated phenolics, however, are known to have low antimicrobial activity, as was demonstrated in the work by Abreu and co-workers, in which apigenin, chrysin, daidzein, and genistein were inactive against different strains of MRSA with MICs $>120 \mu\text{g mL}^{-1}$.³⁵ Therefore, EtOAc extraction leads to more selective extraction of antimicrobial prenylated phenolics for liquorice roots valorisation.

Content of species-specific marker compounds glab (**G-I-11**, *G. glabra*), licoA (**I17**, *G. inflata*), and glycy (**U9**, *G. uralensis*) was 8.7 ± 0.7 , 23.7 ± 1.1 , and $18.5 \pm 1.3 \mu\text{g per g DW extract}$, respectively (corresponding to 0.24 ± 0.02 , 0.54 ± 0.02 , and $0.86 \pm 0.06 \mu\text{g per g dry roots}$) (Table H1, ESI†). These results are in line with reported average contents of 0.028–1.0, 0.3–3.8, and 0.018–1.1 $\mu\text{g per g DW roots}$ for glab, licoA, and glycy in 95 batches of *G. glabra*, *G. inflata*, and *G. uralensis*, respectively.¹⁴ These results indicate that liquorice spent is an equally rich source in prenylated phenolics compared to liquorice roots.

Special attention was given to peaks **I20** (licoisoflavone B or sophoraisoflavone A or semilicoisoflavone B) in *G. inflata* and **U22** (licoricidin) in *G. uralensis* (Fig. 2), as these prenylated compounds showed the highest peak intensity at 280 nm.

Content of **I20** and **U22** was 23.7 ± 1.7 and $32.6 \pm 2.1 \mu\text{g per g DW extract}$ (corresponding to 0.54 ± 0.04 and $1.51 \pm 0.1 \mu\text{g per g DW roots}$) (Table H1, ESI†), respectively. For peak **I20**, we could not differentiate between B-ring 2,2-dimethylpyran (2,2-DMP) ring prenylated isomers licoisoflavone B, sophoraisoflavone A, and semilicoisoflavone B with our recently developed prenylation guideline.¹³ Licoisoflavone B, sophoraisoflavone A, and semilicoisoflavone B have been identified and quantified in *G. inflata* before, with contents ranging between 9–463, 1.0–16, and 0.21–31 $\mu\text{g per g DW roots}$, respectively. Also licoricidin has been identified and

quantified in *G. uralensis* roots, with contents ranging between 11.1–1507 $\mu\text{g per g DW roots}$.¹⁴

Analysis of phenolic compound distribution in the EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* showed that the majority of compounds belonged to the isoflavonoids (Fig. 3A), with 52 ± 4.3 , 93 ± 4.8 , and $117 \pm 6.9 \text{ mg per g DW extract}$, respectively (or 49 ± 1.9 , 50 ± 0.24 , and $60 \pm 0.20\% (w/w)$ extract). With respect to subclass, *G. glabra* and *G. uralensis* were particularly rich in isoflavans with contents of 32 ± 3.0 and $48 \pm 3.2 \text{ mg per g DW extract}$, respectively (or $31 \pm 0.93\%$ and $25 \pm 0.26\% (w/w)$ extract), and isoflavones representing the majority in *G. inflata* with $73 \pm 4.0 \text{ mg per g DW extract}$ (or $39 \pm 0.12\% (w/w)$ extract). Surprisingly, our results demonstrate that chalcones were not particularly abundant in *G. inflata* ($29 \pm 1.2 \text{ mg per g DW extract}$ or $15 \pm 0.21\% (w/w)$ extract), as was shown by Song and co-workers, in which approximately 50% of non-glycosylated prenylated phenolics in *G. inflata* were chalcones. This difference might be explained by batch variability (e.g. differences in harvest time, origin, cultivation).¹⁴

Further analysis focused on prenylation (Fig. 3B) showed that *G. glabra* was abundant in double prenylated compounds with $55 \pm 8.4 \text{ mg per g DW extract}$ (or $52 \pm 1.7\% (w/w)$ extract), and *G. inflata* and *G. uralensis* were rich in single prenylated compounds with 93 ± 4.7 and $86 \pm 6.3 \text{ mg per g DW extract}$, respectively (or $50 \pm 0.15\%$ and $44 \pm 0.52\% (w/w)$ extract). Regarding prenyl configuration, the majority of double prenylated compounds in *G. glabra* were double 3,3-dimethylallyl (3,3-DMA) chain prenylated ($35 \pm 6.8 \text{ mg per g DW extract}$, or $33 \pm 2.5\% (w/w)$ extract). The single prenylated compounds in *G. inflata* were equally 3,3-DMA chain or 1,1-dimethylallyl (1,1-DMA) chain (with $46 \pm 1.8 \text{ mg g}^{-1}$ chain prenylated or $24 \pm 0.34\% (w/w)$ extract) and 2,2-DMP ring prenylated (with $47 \pm 2.9 \text{ mg per g ring prenylated}$ or $25 \pm 0.22\% (w/w)$ extract).

Table 1 Tentative identification of the major prenylated phenolics in EtOAc extracts of *G. glabra*, *G. inflata*, and *G. uralensis* spent and the spectroscopic and spectrometric data obtained in positive ionisation (PI) mode in ESI-IT-MSⁿ and ESI-FT-MS. Numbers refer to peaks in Fig. 2. Estimated content of prenylated phenolics is shown in mg per g DW liquorice spent extract

No. ^a	Identified compound (Subclass)	UV _{max} ^b (nm)	Molecular formula	IT-MS ⁿ			FT-MS		
				[M + H] ⁺ (m/z) (R.A.)	MS ² PI mode m/z (R.A.)	MS ³ PI mode m/z (R.A.)	[M + H] ⁺ m/z calculated	[M + H] ⁺ m/z observed	Error (ppm)
G-I-11	Glabridin (<i>Isoflavan</i>)	<u>278</u> , 322	C ₂₀ H ₂₀ O ₄	325	189 (100), 203 (18)	147 (100), 171 (24)	325.14345	325.14335	-0.26
G18	Kanzonol Y/Glycybridin C (2'- hydroxy-dihydrochalcone)	282, 338	C ₂₅ H ₃₀ O ₅	411	175 (34), 337 (16), 355 (19), 365 (9), 393 (100)	175 (100), 337 (33), 365 (11)	411.21660	411.21698	0.92
G33	Hispaglabridin A (<i>Isoflavan</i>)	<u>278</u> , 330	C ₂₅ H ₂₈ O ₄	393	191 (72), 337 (100)	149 (23), 161 (16), 173 (12), 177 (10), 187 (37), 189 (100), 201 (32), 203 (22), 295 (22), 319 (10)	393.20604	393.20609	0.14
G39	Hispaglabridin B (<i>Isoflavan</i>)	278	C ₂₅ H ₂₆ O ₄	391	147 (8), 189 (100)	147 (100), 171 (22)	391.19038	391.19034	5.10 ± 0.49
G-I-9	Glabrocoumarin (3'- Arylcoumarin)	350	C ₂₀ H ₁₆ O ₅	337	267 (11), 283 (34), 295 (100), 296 (10), 309 (41), 319 (41)	137 (12), 239 (78), 267 (100), 268 (10), 277 (8), 295 (87)	337.10704	337.10699	-0.018
I-I-7	Licochalcone A (<i>Chalcone</i>)	<u>314</u> , <u>378</u>	C ₂₁ H ₂₂ O ₄	339	219 (10), 245 (21), 271 (30), 297 (100)	191 (100), 203 (31), 227 (27), 229 (17), 237 (17), 241 (11), 255 (21), 265 (22)	339.15907	339.15933	0.72
I-U-20	Licoisoflavone B/ Sophoraisoflavone A/ Semilicoisoflavone B (<i>Isoflavone</i>)	262	C ₂₀ H ₁₆ O ₆	353	299 (20), 311 (100), 325 (30), 335 (22)	153 (24), 255 (58), 283 (100), 284 (10), 293 (12), 311 (54)	353.10197	353.10184	-0.35
G-I-23	Glabrol (<i>Flavanone</i>)	<u>282</u> , 318	C ₂₅ H ₂₈ O ₄	393	205 (13), 337 (100)	177 (27), 191 (16), 195 (52), 203 (17), 213 (100), 281 (19), 295 (22), 319 (63), 322 (27)	393.20604	393.20599	-0.12
U7	n.i. O-3,3-DMA ^d (<i>Chalcone</i>)	370	C ₂₁ H ₂₂ O ₅	355	287 (100), 299 (70)	165 (13), 231 (11), 241 (14), 287 (100)	355.15400	355.15396	-0.11
U9	Glycycoumarin (3-Arylcoumarin)	350	C ₂₁ H ₂₀ O ₆	369	285 (45), 301 (5), 313 (100), 314 (13), 327 (14), 341 (11)	149 (19), 191 (19), 209 (11), 243 (17), 257 (10), 271 (27), 281 (23), 283 (32), 285 (100), 298 (14)	369.13325	369.13330	0.10
U22	Licoricidin (<i>Isoflavan</i>)	<u>282</u> , 338	C ₂₆ H ₃₂ O ₅	425	191 (11), 221 (19), 369 (100)	167 (85), 189 (100), 191 (17), 219 (17), 233 (17), 313 (45), 351 (13)	425.23225	425.23178	1.11
U31	Licorisoflavan A (<i>Isoflavan</i>)	<u>282</u> , 342	C ₂₇ H ₃₄ O ₅	439	371 (47), 383 (100)	181 (100), 189 (86), 191 (20), 193 (15), 215 (12), 327 (58)	439.24791	439.24805	0.34
									10.04 ± 0.61

R.A. = Relative abundance. ^a No = number corresponds to peaks in Fig. 2 and tentatively identified compounds in Tables E1 and F1 (ESI[†]). ^b Underlined numbers indicate the main UV absorbance peak. ^c Estimated content of all tentatively identified compounds is shown in Table H1 (ESI[†]). ^d 3,3-DMA = 3,3-dimethylallyl chain.

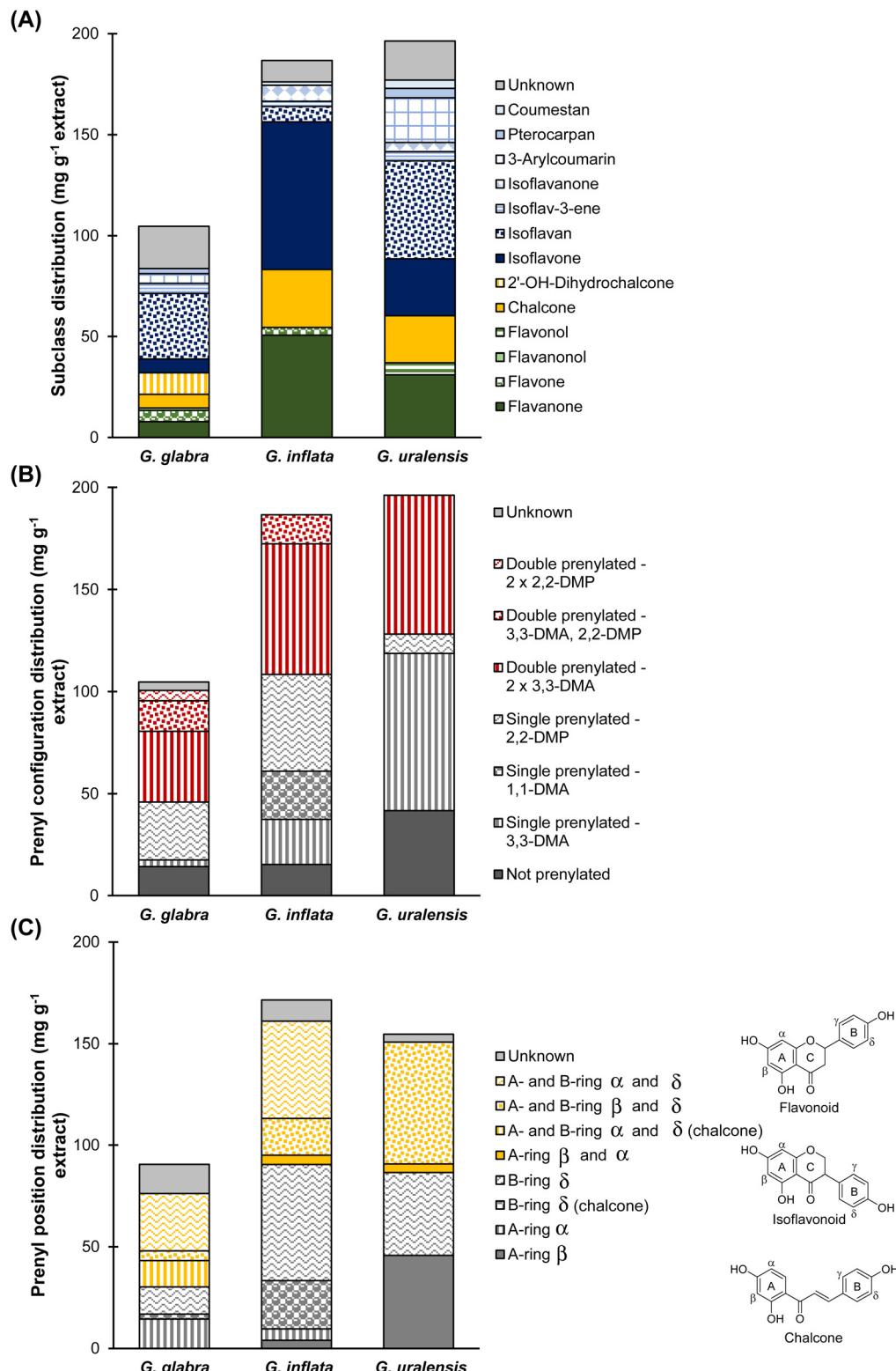


Fig. 3 Weight distribution of (A) phenolic subclass, (B) prenyl configuration, and (C) prenyl position in EtOAc spent extracts *Glycyrrhiza glabra*, *G. inflata*, and *G. uralensis*.

Lastly, the majority of single prenylated compounds in *G. uralensis* were 3,3-DMA chain prenylated (77 ± 5.5 mg per g DW extract or $39 \pm 0.41\%$ (w/w) extract). Regarding the prenyl

position (Fig. 3C), the majority of double prenylated compounds in *G. glabra* were A- and B-ring prenylated with 46 mg per g DW extract (or $48 \pm 1.1\%$ (w/w) extract); the majority of

single prenylated compounds in *G. inflata* were B-ring prenylated with 81 ± 4.3 mg per g DW extract (or $47 \pm 0.14\%$ (w/w) extract), and the single prenylated compounds in *G. uralensis* were equally A- and B-ring prenylated with 46 ± 4.1 and 41 ± 2.3 mg per g DW extract, respectively (or $30 \pm 0.62\%$ and $26 \pm 0.34\%$ (w/w) extract) (Fig. 3C).

To conclude, spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* showed a distinct compositional profile (Fig. 2), in which species-specific compounds glab (*G. glabra*), licoA (*G. inflata*), and glycy (*G. uralensis*) were the main prenylated phenolics present, with 8.7 ± 0.7 , 23.7 ± 1.1 , and 18.5 ± 1.3 mg per g DW extract, respectively, next to licoisoflavone B or sophoraisoflavone A or semilicoisoflavone B with 23.7 ± 1.7 mg per g DW spent extract in *G. inflata*, and licoricidin with 32.6 ± 2.1 mg per g DW spent extract in *G. uralensis*.

3.2. Antimicrobial activity of liquorice spent extracts and species-specific markers glabridin, licochalcone A, and glycy coumarin

EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* and species-specific marker compounds glab, licoA, and glycy were tested for their antimicrobial activity against *L. buchneri*, *Z. parabaili*, *Y. lipolytica*, *S. mutans*, *S. aureus*, and *E. coli*. Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) are shown in Table 2.

3.2.1. Antibacterial activity. Liquorice spent extracts and marker compounds glab, licoA, and glycy showed antibacterial activity against the Gram-positive bacteria *S. aureus*, *S. mutans*, and *L. buchneri*, whereas no activity was seen against the Gram-negative bacterium *E. coli* (Table 2). For example, liquorice spent extracts inhibited growth of *S. aureus*, with MICs of 75, 25–50, and 25 $\mu\text{g mL}^{-1}$ for *G. glabra*, *G. inflata*, and *G. uralensis*, respectively (with log reductions (LRs) of 3.7 ± 0.01 , 1.9 ± 2.8 , and 1.9 ± 1.7). At these MICs, the content of prenylated phenolics (minimum inhibitory prenylated phenolics concentration, or MIPPC, (Table I1, ESI†)) was $7.2 \mu\text{g mL}^{-1}$ for *G. glabra*, $4.3\text{--}8.6 \mu\text{g mL}^{-1}$ for *G. inflata*, and $3.9 \mu\text{g mL}^{-1}$ for *G. uralensis*. In contrast, liquorice spent extracts did not show antibacterial activity up to $2000 \mu\text{g mL}^{-1}$ against different *E. coli* strains (with MIPPCs >192 , >342 , and $>310 \mu\text{g mL}^{-1}$ for *G. glabra*, *G. inflata*, and *G. uralensis*, respectively, Table I1†). The same trend was observed for the marker compounds. Glab, licoA, and glycy showed anti-*S. aureus* activity with MICs between $12.5\text{--}25 \mu\text{g mL}^{-1}$ (with LRs of 3.3 ± 1.3 , 3.7 ± 2.4 , and 0.9 ± 0.6), which is associated with good activity,¹⁹ whereas no anti-*E. coli* activity was seen up to $200 \mu\text{g mL}^{-1}$ (Table 2). Gram-negative bacteria are known to have higher intrinsic resistance towards most antimicrobials than Gram-positive bacteria, caused by (i) the limited permeability by the highly ordered and crystalline lipopolysaccharides (LPS) in the outer bacterial membrane, (ii) size restrictions of hydrophilic porins (generally $M_w < 600\text{--}700$), and (iii) effective excretion of antimicrobials across the double membrane by double-membrane spanning efflux pumps.^{33,36} Addition of efflux pump inhibitor PAβN did not increase susceptibility towards the

liquorice spent extracts up to $2000 \mu\text{g mL}^{-1}$ (data not shown). However, addition of PAβN increased susceptibility of *E. coli* K12 towards glab and glycy, as MICs decreased from $>100 \mu\text{g mL}^{-1}$ to $12.5\text{--}25 \mu\text{g mL}^{-1}$, respectively (Table 2). These results demonstrate that glab and glycy can enter the outer membrane of *E. coli*, and that intrinsic resistance of *E. coli* was due to active efflux rather than limited influx. As the liquorice spent extracts are rich in prenylated phenolics, we propose that the extracts interact with the bacterial cytoplasmic membrane, thereby changing membrane fluidity, which can lead to membrane permeabilization, and bacterial cell death.^{20,37,38}

Large differences in susceptibility (MICs) between Gram-positive bacteria towards the liquorice spent extracts were observed (Table 2). *S. aureus* was the most susceptible towards the spent extracts, with MICs between 25 and $75 \mu\text{g mL}^{-1}$, whereas MICs against *S. mutans* were ranging between $100 \mu\text{g mL}^{-1}$ for *G. inflata*, $250 \mu\text{g mL}^{-1}$ for *G. uralensis*, and $>1000 \mu\text{g mL}^{-1}$ for *G. glabra*. Liquorice spent extracts showed bactericidal activity against *S. aureus*, as the ratio MBC/MIC was <4 (Table 2).³⁹ In contrast, *G. inflata* spent extract was bacteriostatic against *S. mutans*, shown by the ratio MBC/MIC >4 .³⁹ The underlying mechanism behind this increased susceptibility of *S. aureus* compared to *S. mutans* is unknown. Possibly, the production of a glue-like extracellular polymer glucan (that promotes formation of a biofilm) in *S. mutans* provides an extra barrier against the prenylated phenolics by complicating the interaction with the bacterial cell membrane.⁴⁰

In some cases, antibacterial activity of marker compounds was found to correlate with the antibacterial activity of the liquorice spent extracts; for example, the MIPPC of *G. inflata* against *S. mutans* was $17.1 \mu\text{g mL}^{-1}$ (Table I1, ESI†), and the MIC of licoA was $12.5 \mu\text{g mL}^{-1}$ (Table 2). This might hint that the marker prenylated phenolic in *G. inflata* spent extract is the main contributor to its antibacterial activity. In contrast, we observed that the MIPPC of *G. uralensis* extract against *S. aureus* ($3.9 \mu\text{g mL}^{-1}$) was about 3-fold lower than the MIC of the marker glycy ($12.5 \mu\text{g mL}^{-1}$) (Table I1,† Table 2). This might be explained by other potent antibacterial prenylated phenolics in our EtOAc spent extract of *G. uralensis*. For example, the double chain prenylated isoflavone 6,8-diprenylgenistein (4.2 ± 0.2 mg per g DW extract, Table H1, ESI†) has shown anti-MRSA activity with a MIC of $9 \mu\text{g mL}^{-1}$,³⁷ and the double chain prenylated isoflavan licorisoiflavan A (10.0 ± 0.6 mg per g DW extract, Table H1†) showed a MIC between $1\text{--}4 \mu\text{g mL}^{-1}$ against different species of *S. mutans*.⁴¹ We cannot rule out that prenylated phenolics show synergistic effects with each other, as was shown by Chen and co-workers,⁴² or with other minor or unknown compounds (small amounts of non-prenylated phenolics and triterpenoid aglycones were found in the liquorice spent extracts). However, the identified non-prenylated phenolics (e.g. **G-I-U-1** to **G-I-U-3**, Table H1, ESI†) and triterpenoid (**G37**, Table H1†) are not expected to significantly contribute to the antibacterial activity observed.^{7,35,43,44}

In summary, *G. glabra*, *G. inflata*, and *G. uralensis* spent extracts and marker compounds glab, licoA, and glycy showed



Table 2 Minimum inhibitory concentrations (MIC) and minimum bactericidal/fungicidal concentrations (MBC/MFC) in $\mu\text{g mL}^{-1}$ of liquorice spent extracts and marker compounds against various microorganisms

Bacteria/ yeasts	<i>G. glabra</i>		<i>G. inflata</i>		<i>G. uralensis</i>		Glabridin		Licochalcone A		Glyceoumarin	
	MIC (LR ^a \pm SD ^b)	MBC/MFC (LR \pm SD)	MIC (LR \pm SD)	MBC/MFC (LR \pm SD)	MIC (LR \pm SD)	MBC/MFC (LR \pm SD)	MIC (LR \pm SD)	MBC/MFC (LR \pm SD)	MIC (LR \pm SD)	MBC/MFC (LR \pm SD)	MIC (LR \pm SD)	MBC/MFC (LR \pm SD)
BACTERIA												
<i>L. buchneri</i>												
ATCC 4005	250–500 (2.22 \pm 1.54)	250–1000 (3.14 \pm 0.79)	250 (3.55 \pm 0.98)	250 (3.55 \pm 2.54)	50 (2.66 \pm 0.98)	50–100 (4.65 \pm 2.54)	12.5–25 (2.33 \pm 0.78)	12.5–25 (2.33 \pm 0.28)	12.5 (4.47 \pm 1.47)	12.5–25 (5.07 \pm 0.48)	25 (4.04 \pm 1.54)	25 (4.04 \pm 1.54)
L4	250–500 (1.59 \pm 1.33)	250–1000 (3.56 \pm 1.34)	250 (4.48 \pm 1.12)	250 (4.48 \pm 1.12)	50–100 (3.94 \pm 3.94)	50–100 (3.57 \pm 3.94)	12.5–25 (2.57 \pm 2.27)	25 (5.13 \pm 0.39)	3.125–6.25 (3.75 \pm 1.39)	3.125–6.25 (3.75 \pm 1.39)	12.5 (4.80 \pm 0.55)	12.5 (4.80 \pm 0.55)
<i>S. mutans</i>	>1000 (n.d.)	>2000 (n.d.)	100 (0.59 \pm 0.35)	>1000 (n.d.)	250 (0.75 \pm 0.12)	1000 (2.58 \pm 0.12)	12.5 (0.98 \pm 0.08)	25–50 (3.62 \pm 1.20)	12.5 (0.72 \pm 0.09)	>100 (n.d.)	25 (1.60 \pm 0.13)	50 (4.52 \pm 0.40)
<i>S. aureus</i>												
ATCC 27175	75 (3.71 \pm 0.01)	75 (3.71 \pm 0.01)	25–50 (1.86 \pm 2.80)	50–100 (4.88 \pm 1.34)	25–50 (1.92 \pm 1.75)	25–50 (3.61 \pm 1.25)	12.5–25 (3.31 \pm 0.83)	12.5–25 (4.35 \pm 2.42)	12.5 (3.68 \pm 2.42)	12.5–25 (4.88 \pm 0.34)	12.5 (0.93 \pm 0.59)	16.7–25 (4.35 \pm 0.77)
<i>E. coli</i>												
K12	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>200 (n.d.)	>200 (n.d.)	>200 (n.d.)	>200 (n.d.)
O54:H21	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>100 (n.d.)	12.5–25 ^d (5.23 \pm 0.08)	12.5–25 ^d (5.23 \pm 0.08)	>100 ^d (n.d.)	>100 ^d (n.d.)
O88:H8	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)
YEASTS												
<i>Y. lipolytica</i>												
Food isolate	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>25–<50 (4.94 \pm 0.23)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)	100 (4.90 \pm 0.17)	100 (4.90 \pm 0.17)
<i>Z. parabailii</i>												
ATCC 60483	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>12.5–<25 (4.34 \pm 1.29)	25 (4.34 \pm 1.29)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)	>100 (n.d.)
UL 3699	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	>2000 (n.d.)	12.5–25 (3.62 \pm 2.67)	25 (5.24 \pm 0.15)	>100 (n.d.)	>100 (n.d.)	100 (4.19 \pm 0.98)	100 (4.19 \pm 0.98)

^a LR = log reduction after 24 h incubation from initial inoculum. ^b SD = standard deviation. ^c n.d. = not determined. ^d In the presence of efflux pump inhibitor PAPN.

antibacterial activity against Gram-positive bacteria *L. buchneri*, *S. mutans*, and *S. aureus*. The order of antibacterial potency for the extracts was *G. uralensis* (MICs 25–250 µg mL⁻¹) ≈ *G. inflata* (MICs 25–250 µg mL⁻¹) > *G. glabra* (MICs 75 → 1000 µg mL⁻¹), whereas marker compounds were equally potent (MICs 12.5–25 µg mL⁻¹). No antibacterial activity was observed against the Gram-negative bacterium *E. coli* up to 2000 µg mL⁻¹ for the liquorice spent extracts, and 200 µg mL⁻¹ for marker compounds, without presence of efflux pump inhibitor PAβN. Antibacterial activity of glab and glycy was observed upon addition of PAβN (as MICs reduced from >200 to 12.5–25 µg mL⁻¹).

3.2.2. Antifungal activity. Liquorice spent extracts did not show antifungal activity against *Z. parabailli* and *Y. lipolytica*, with MICs >2000 µg mL⁻¹ of crude extract (MIPPCs of >192, >342, and >310 µg mL⁻¹ for *G. glabra*, *G. inflata*, and *G. uralensis*, respectively) (Table 2 and Table I1, ESI†). However, at 2000 µg mL⁻¹, *G. glabra*, *G. inflata*, and *G. uralensis* spent extracts showed delay in fungal growth of *Y. lipolytica*, with growth delay ratios of 2.8 ± 1.6, 2.7 ± 0.7, and 3.0 ± 1.3, as is shown in Table K1 (ESI†). Against *Z. parabailli*, this growth delay ratio was less pronounced, with maximum delays for *G. uralensis* (ratio 2.0 ± 0.1 for ATCC 60483 and ratio 2.8 ± 0.1 for UL 3699), and no growth delay for *G. glabra* (ratio 1.3 ± 0.1 for ATCC 60483 and 1.1 ± 0.1 for UL 3699). Aforementioned higher growth delay ratio's in *G. uralensis* extract might be explained by the higher content of prenylated phenolics in the extract (155 vs. 96 mg g⁻¹ prenylated phenolics DW extract for *G. uralensis* and *G. glabra*, respectively) (section 3.1). Additionally, *G. uralensis* extract is rich in single prenylated compounds, whereas *G. glabra* is more abundant in double prenylated compounds. It was recently shown by Kalli and co-workers that single prenylated (iso)flavonoids possess superior anti-*Z. parabailli* activity compared to double prenylated (iso)flavonoids (section 3.1).⁴⁵ All growth delay ratio's at the different concentrations tested are shown in Table K1 (ESI†).

Glab showed good antifungal activity against *Z. parabailli*, with a MIC between 12.5–25 µg mL⁻¹, which corresponded to the reported MIC (against *Z. parabailli*) of glab between 6.25–12.5 µg mL⁻¹.⁴⁵ In contrast, licoA and glycy showed weak antifungal activity, with MICs of >100 µg mL⁻¹ and 100 µg mL⁻¹, respectively. When comparing antifungal activity of the marker compounds with the most potent natural agent against *Z. parabailli*, polygodial (MIC 50 µg mL⁻¹), fungicidal activity of glab was 2–4× higher.⁴⁶ Against *Y. lipolytica*, the species-specific marker compounds were moderately active to inactive, with a MIC of 50 µg mL⁻¹ for glab, >100 µg mL⁻¹ for licoA, and 100 µg mL⁻¹ for glycy, respectively.

Recently, it was proposed that antifungal activity of prenylated (iso)flavonoids was associated with *i.a.* their overall hydrophobicity and the location of hydrophobic moieties, which are involved in fungal membrane permeabilization and consequently fungal cell death.⁴⁵ The same authors suggested that prenylated (iso)flavonoids with a log *D* (lipophilicity) value ≤4.5 possess antifungal activity, whereas very hydrophobic

molecules (log *D* > 5.0) were inactive. Possibly, hydrophobic molecules, such as licoA (with a MIC of >100 µg mL⁻¹ and a log *D* value of 4.8, Table J1, ESI†), are not able to cross the complex hydrophilic cell wall of yeasts. The location of hydrophobic moieties (described with the hydrophobic integy moment, Table J1†) was also shown to be important for effective hydrophobic interactions with the lipid core of the fungal membrane.⁴⁵ Potent antifungal prenylated (iso)flavonoids showed a hydrophobic integy moment >0.9 Å.⁴⁵ Besides the higher hydrophobicity, this could also explain the inactivity of licoA, which has a hydrophobic integy moment of 0.5 Å (Table J1, ESI†). The difference in antifungal activity between glab (MIC 12.5–25 µg mL⁻¹, Table 2) and glycy (100 µg mL⁻¹, Table 2) can possibly be explained by their flexibility. We previously postulated that flexible prenylated (iso)flavonoids interact more effectively inside a bacterial membrane than less flexible prenylated (iso)flavonoids, leading to disruption of membrane integrity.²³ In the present study, similarly to that of a set of prenylated (iso)flavonoids tested against *Z. parabailli* (Table J1, ESI†),⁴⁵ the opposite effect was observed. Less flexible compounds, such as glab (with a flexibility index of 2.9) or dehydroglyceollin I (a pterocarpene with a flexibility index of 2.2 and a MIC of 12.5–25 µg mL⁻¹, Table J1†) were more potently antifungal than more flexible compounds, such as glycy (with a flexibility index of 4.1) or 6'-prenylpiscidone (a isoflavone with a flexibility index of 5.9 and a MIC of >>25 µg mL⁻¹, Table J1†). However, this does not explain the inactivity (>100 µg mL⁻¹) of licoA with a flexibility index of 4.2. We suggest that a proper balance between hydrophobicity, hydrophobic integy moment, and flexibility is considered optimal for effective interactions with the fungal membrane.^{45,47}

To summarize, the liquorice spent extracts did not show antifungal activity against *Z. parabailli* and *Y. lipolytica* at the highest concentrations tested (MIC >2000 µg mL⁻¹), although fungal growth delay (ratios ranging between 1.1–2.5), especially for *G. uralensis*, was seen at 2000 µg mL⁻¹ (with a ratio between 2.0–2.8). Marker compound glab showed good activity against *Z. parabailli* (MIC 12.5–25 µg mL⁻¹) and moderate activity against *Y. lipolytica* (MIC 50 µg mL⁻¹). Overall, these prenylated compounds showed higher or similar anti-yeast activity than the reported natural agent polygodial.

3.3. Cytotoxicity and effects on cell viability of liquorice spent extracts and species-specific markers glabridin, licochalcone A, and glycy coumarin

To evaluate potential effect of liquorice spent extracts and species-specific markers on cell cytotoxicity and viability, the release of LDH (Fig. 4A and B) and cellular mitochondrial dehydrogenase activity (WST-1) (Fig. 4C and D), respectively, were measured in human colorectal adenocarcinoma cells (Caco-2).

3.3.1. Cytotoxicity and cell viability of Caco-2 cells after exposure to liquorice spent extracts. Spent extract of *G. glabra* did not show any cytotoxicity up to the highest concentration tested compared to the negative control (growth medium)



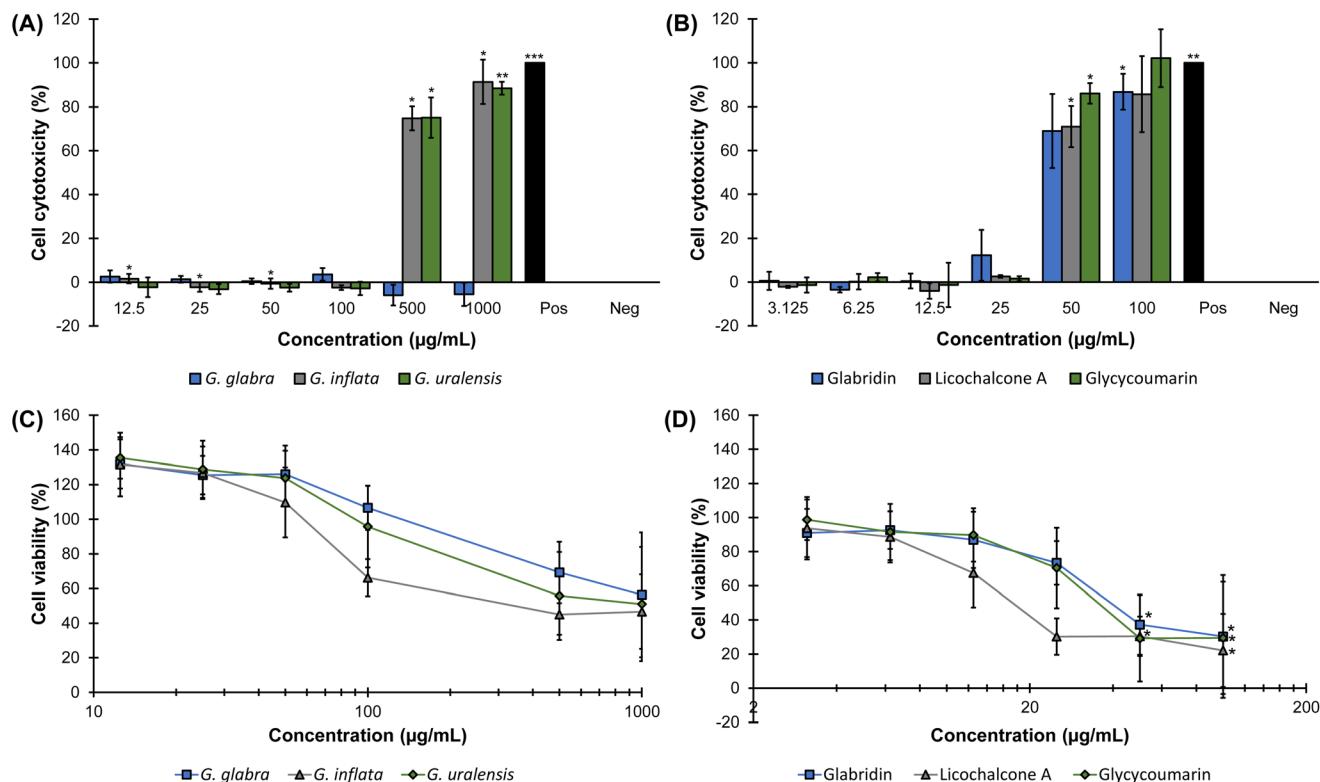


Fig. 4 Cell cytotoxicity (A and B) and cell viability (C and D) of (A and C) EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis* and (B and D) species-specific marker compounds glabridin, licochalcone A, and glycoumarin. Values are means \pm standard deviation, measured in triplicate in three biological replicates. Cell cytotoxicity and cell viability is compared with the negative control (cells with growth medium, set at 0% for cytotoxicity and 100% for cell viability). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

(1000 $\mu\text{g mL}^{-1}$ crude extract, MIPPC 96 $\mu\text{g mL}^{-1}$) (Fig. 4A). In contrast, spent extracts of *G. inflata* ($p = 0.023$) and *G. uralensis* ($p = 0.048$) were cytotoxic at 500 $\mu\text{g mL}^{-1}$ crude extract with 75% cytotoxicity for both *G. inflata* and *G. uralensis* (MIPPCs 85.5 $\mu\text{g mL}^{-1}$ and 77.5 $\mu\text{g mL}^{-1}$) (Fig. 4A). Cell viability of Caco-2 cells was affected at lower concentrations than cell cytotoxicity after exposure to the liquorice spent extracts. WST-1 determines metabolic activity of cells and as such is more sensitive than LDH, which is a measure of cell membrane damage. A trend of decreased cell viability was observed after exposure to *G. glabra* and *G. uralensis* spent extracts, with a decrease from 107% to 69% for *G. glabra* and from 96% to 56% for *G. uralensis* at 100 and 500 $\mu\text{g mL}^{-1}$ crude extract (MIPPCs 9.1–45.5 and 15.5–77.5 $\mu\text{g mL}^{-1}$) (Fig. 4C). For *G. inflata* spent extract, a decreased trend in cell viability from 110% to 66% was observed between 50 and 100 $\mu\text{g mL}^{-1}$ (MIPPC 8.6–17.1 $\mu\text{g mL}^{-1}$). Colour was visually observed at the highest concentrations of the liquorice spent extracts ($>100 \mu\text{g mL}^{-1}$) compared to Caco-2 cells with growth medium alone. Therefore, we verified whether colour affected cell cytotoxicity and viability measurements (Fig. L1, ESI†). Cell cytotoxicity and cell viability measurements were not significantly affected by the coloured spent extracts up to 1000 $\mu\text{g mL}^{-1}$ crude spent extract. Recently, Molčanová *et al.* reviewed effects of 394 prenylated phenolics with potential cytotoxic activity against

cancer cells and summarized suggested structure–activity relationships (SAR) of prenylated phenolics.²⁷ Generally (i) addition of a prenyl moiety increased cytotoxicity,^{48,49} (ii) single prenylated compounds showed higher cytotoxicity than double prenylated compounds,⁵⁰ (iii) ring prenylated compounds (pyran [e.g. 2,2-DMP] and furan) were more cytotoxic than chain (e.g. 3,3-DMA) prenylated compounds,⁵⁰ (iv) flavones and flavonols were more cytotoxic than flavanones and chalcones,⁵¹ and (v) prenylation at *C*8 (or α position, Fig. 3C) showed higher cytotoxicity than prenylation at position *C*6 (β) on the A-ring.⁵¹ The high percentage of prenylation (92%, Fig. 3B) in *G. inflata* spent extract of which a large part is single prenylated (50%, Fig. 3B) could explain its relatively high cytotoxicity compared to *G. glabra* and *G. uralensis* spent extracts (Fig. 4A and C). However, *G. inflata* spent extract is neither rich in flavones (2%) and flavonols (not detectable), nor rich in *C*8 (β) prenylated compounds (3%), but is relatively rich in flavanones (27%) and chalcones (15%), and *C*3' (γ) prenylated compounds (33%) (Fig. 3). We hypothesize that other substitutions on the phenolic backbone, *i.e.* hydroxylation and methoxylation also affect cytotoxicity (and cell viability) of prenylated phenolics, as was shown by Daskiewicz and co-workers.⁵¹

Previously, it was shown that *G. glabra* solvent-based root extracts were cytotoxic between 7.4–34 $\mu\text{g mL}^{-1}$ in colorectal cancer cells.⁵² Additionally, Jo *et al.* evaluated cytotoxicity of



G. uralensis root extracts and reported antiproliferative effects at 50 $\mu\text{g mL}^{-1}$ in a breast cancer cell line, which was caused by apoptosis due to cell cycle arrest.⁵³ Prenylated phenolics were not quantified in these extracts. However, aforementioned root extracts^{52,53} showed cytotoxicity at lower concentrations than observed in our study (we observed cytotoxicity at 500 $\mu\text{g mL}^{-1}$ for *G. inflata* and *G. uralensis*, and >1000 $\mu\text{g mL}^{-1}$ for *G. glabra*, MIPPCs of 85.5 $\mu\text{g mL}^{-1}$, 77.5 $\mu\text{g mL}^{-1}$, and >96 $\mu\text{g mL}^{-1}$, respectively, Fig. 4A). Differences in cytotoxicity could be explained by differences in prenylated phenolics content of the extracts, incubation time, and cell line.

An increased trend in apparent cell viability (or metabolic activity) in Caco-2 cells after exposure to the liquorice spent extracts <50 $\mu\text{g mL}^{-1}$ was seen. For example, exposure to 12.5 $\mu\text{g mL}^{-1}$ crude liquorice spent extracts increased metabolic activity 32%, 32%, and 35% for *G. glabra*, *G. inflata*, and *G. uralensis*, respectively (Fig. 4C). It is unknown why apparent cell viability at low concentrations was increased, but it could be hypothesized this was caused by other compounds in the liquorice spent extracts, since we did not observe an upward trend in metabolic activity for glab, licoA, and glycy (Fig. 4D). Another hypothesis is that this observed trend is an early indication of apoptosis, which is an active process that coincides with apparently increased metabolic activity. However, in general it has been shown that prenylated phenolics show anti-proliferative effects and induce cell cytotoxicity in cancer cell lines.^{24,27,54} Interestingly, for some prenylated chalcones such as xanthohumol, the concentrations for these cytotoxic effects were much higher in primary cell lines in comparison to cancer cell lines.^{24–26}

3.3.2. Cytotoxicity and cell viability of Caco-2 cells after exposure to glabridin, licochalcone A, and glycy coumarin. Cytotoxicity in Caco-2 cells after exposure to the species-specific marker compounds glab ($p = 0.18$), licoA ($p = 0.035$), and glycy ($p = 0.019$) was observed at 50 $\mu\text{g mL}^{-1}$ with a distinct off-on effect, with 69%, 71%, and 86% cytotoxicity, respectively, while there was no cytotoxicity measured at 25 $\mu\text{g mL}^{-1}$ (cytotoxicity < 20%, $p > 0.05$) (Fig. 4B). All standards showed a similar trend ($p > 0.05$), where cell viability decreased to 73% at 25 $\mu\text{g mL}^{-1}$ (77 μM) for glab, to 70% at 25 $\mu\text{g mL}^{-1}$ (68 μM) for glycy, and to 37% at 12.5 $\mu\text{g mL}^{-1}$ (37 μM) for licoA (Fig. 4D). At 12.5 $\mu\text{g mL}^{-1}$ for glab and glycy, and at 6.25 $\mu\text{g mL}^{-1}$ for licoA, no effects on cell viability were measured (viability >80% compared to the negative control, $p > 0.05$). Our observed cytotoxicity is in line with reported cytotoxicity of glab against other cell lines.^{52,55} Cytotoxic mode of action of structural similar compounds 4'-OH-methylglabridin and glabrene was related to changes in cell cycle signalling pathways, ultimately leading to apoptotic cell death via different mechanisms.^{52,56} It could be hypothesized that, due to structural similarity, glab shows a similar cytotoxic mode of action. Our observed cytotoxicity of licoA at 12.5 $\mu\text{g mL}^{-1}$ is in line with previously reported cytotoxicity in different cancer cell lines.^{57,58} Reported cytotoxic mode of action of licoA has been linked to cell cycle arrest that leads to apoptosis.⁵⁹ Lastly, our observed decreased viability (from 90% to 70% cell viabi-

lity compared to the negative control, Fig. 4D) between 12.5 and 25 $\mu\text{g mL}^{-1}$ of glycy is in line with reported cytotoxicity in cancer cells.^{60,61} Besides *in vitro* cytotoxicity in cancer cells, glycy has also shown selective anti-cancer activity in Hep-G2 xenograft mice, due to cell cycle arrest and subsequent apoptosis.⁶¹ It should be noted that we did not take into account possible interactions, absorption, distribution, and metabolism of prenylated phenolics in the gastrointestinal tract. Notably, it was previously shown that there was no significant first-pass metabolism of prenylated phenolics (*i.a.* licoA and licochalcone C) during intestinal absorption in a Caco-2 monolayer model.⁶² Nevertheless, possible interactions (*e.g.* with proteins), but also phase I and II metabolism, including glucuronidation, sulfation, and methylation⁶³ of prenylated phenolics need to be further investigated.

To summarize, our results demonstrate that EtOAc spent extracts of *G. inflata*, and *G. uralensis* were cytotoxic at 500 $\mu\text{g mL}^{-1}$ with reduced cell viability between >100–500 $\mu\text{g mL}^{-1}$ and >50–100 $\mu\text{g mL}^{-1}$, respectively, whereas *G. glabra* spent extract did not show cytotoxicity up to 1000 $\mu\text{g mL}^{-1}$, with reduced cell viability between >100–500 $\mu\text{g mL}^{-1}$. Marker compounds glab, licoA, and glycy showed cytotoxicity with a distinct off-on effect at 50 $\mu\text{g mL}^{-1}$, but cell viability showed a reduced trend at 25 $\mu\text{g mL}^{-1}$ for glab and glycy, and 12.5 $\mu\text{g mL}^{-1}$ for licoA.

4. Conclusions

In this comprehensive study, we have characterized the prenylated phenolics composition, antimicrobial activity, cytotoxicity, and cell viability of EtOAc spent extracts of *G. glabra*, *G. inflata*, and *G. uralensis*, together with their marker compounds glab, licoA, and glycy. *G. uralensis* and *G. inflata* spent extracts were more antibacterially potent than *G. glabra*, possibly due to their higher (approximately 2× higher) content of prenylated phenolics. *G. glabra* spent extract showed lower cytotoxicity than those of *G. inflata* and *G. uralensis*, possibly explained by its lower prenylated phenolics content and higher relative content in double prenylated phenolics. Linking antibacterial activity of the liquorice spent extracts with cell viability showed that MICs against *S. aureus* coincide with concentrations where cell viability was not reduced. For the other tested Gram positive and Gram negative bacteria, and yeasts, MICs concurred at concentrations where cell viability was reduced. To conclude, in this study we show that liquorice spent extracts rich in prenylated phenolics are potent antimicrobials against Gram positive bacteria, however given their cytotoxicity profiles, further in-depth investigating should reveal their future applicability.

Author contributions

Sarah van Dinteren: Conceptualization, methodology, investigation, visualization, writing – original draft, preparation.



Carla Araya-Cloutier: Conceptualization, methodology, writing – review & editing, supervision. Renger Witkamp: Writing – review & editing. Bo van Ieperen: Investigation. Jocelyn Meijerink: Conceptualization, methodology, writing – review & editing, supervision. Jean-Paul Vincken: Writing – review & editing, supervision.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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