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Deciphering the Anti-Listerial Activity and Phytochemical Composition of Licorice Root Extract Using LC-MS/MS in Combination with In Vitro and Computational Evaluations

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Featured Application: Natural products are an endless source of antimicrobial agents against many foodborne pathogenic bacteria. The present work demonstrates the potential of licorice root extract to efficiently inhibit the growth of a panel of *Listeria* strains and serotypes. Results support the utilization of the plant extract as a promising anti-Listeria agent for the food industry. Furthermore, it also demonstrates the combined use of advanced analytical and in silico methodologies to decode the active components of the extract, opening new horizons in the discovery of novel agents to control *Listeria* bacteria.

Abstract: Licorice roots are a rich source of bioactive compounds with multiple biological activities. The objective of this study was to evaluate the inhibitory effects of licorice root extract against a range of *Listeria* strains. In addition, the correlation of its phytochemical composition with antimicrobial properties was also investigated. Thus, the bacteriostatic and bactericidal effects of licorice root extract on seven *Listeria monocytogenes* strains, *L. grayi*, *L. seeligeri*, and *L. ivanovii* were determined. The minimum inhibitory and bactericidal concentrations ranged from 31.3 to 62.5 μg mL^{-1} and from 62.5 to 250 μg mL^{-1}, respectively. The phytochemical composition of the extract was also analyzed using advanced LC-DAD-qTOF-MS; it is composed of fifty-one compounds belonging to different subgroups of flavonoids and triterpenoids. Subsequently, the anti-Listeria potency of the most abundant phytochemicals was determined using the AntiBac-Pred web tool. In silico calculation showed that liquiritin-apioside and licorice glycoside C1/C2 were strong growth inhibitors of *L. monocytogenes*, as their potency was comparable to well-known antibiotic substances. Overall, the present study demonstrates the potent antimicrobial effect of licorice root extract and reveals its active phytochemicals.

Keywords: antimicrobial activity; flavanones; extract; *Glycyrrhiza uralensis*; *Listeria monocytogenes*; molecular docking; phytochemicals

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1. Introduction

Bacterial contamination is the primary cause of food poisoning and food-borne diseases, as well as a cause of losses due to food spoilage [1]. A range of Gram-positive and

Gram-negative bacteria can cause foodborne infection; however, *Listeria monocytogenes* is recognized as a major risk by the food industry. It is a Gram-positive, rod-shaped, foodborne pathogen that is considered the major causative agent responsible for serious diseases in both humans and animals. *L. monocytogenes* can naturally contaminate a variety of refrigerated and ready-to-eat foods, such as milk and dairy products, vegetables, meat, poultry, and seafood products, and its growth is not prevented by low-temperature storage [2,3]. Infections of human or animal hosts result in clinical presentations that range from asymptomatic carriers to septicemia, encephalitis, or abortions. It is noteworthy that, in the EU, it is zoonosis that has the highest case fatality rate of 10% [4]. As an adaptive, environmental organism, *L. monocytogenes* is quite resistant to a range of standard physical and chemical food preservation methods; hence, there has been a growing interest in the application of synthetic and natural compounds to control *L. monocytogenes* in food [5]. In the last decade, numerous plant extracts and essential oils have been evaluated as potential inhibitors of *L. monocytogenes* bacteria, and the anti-listerial potential of the extracts is mostly correlated with their phenolic composition; various mechanisms of action involving membranes, cytoplasm, and genetic material have been proposed for these compounds [6,7].

Licorice is a native bush in Asia and the Mediterranean region that is used in traditional medicines and folk remedies to treat many diseases [8]. Currently, in vitro and in vivo studies have documented the diverse pharmacological properties of licorice, such as anti-inflammatory, anti-tussic and expectorant, anti-ulcerative, antioxidant, antiviral, anticarcinogenic and antimutagenic, hepato-protective, neuroprotective, anti-depressive, estrogenic and androgenic, and antimicrobial activities [9–12]. Regarding antimicrobial activity, polar and supercritical fluid extracts of licorice root have strong inhibitory effects on Gram-positive bacteria and Gram-negative bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Bacillus subtilis*. Based on the above inhibitory activities against bacteria, licorice root extracts may serve as an alternative therapy for treating diverse bacterial infections. Furthermore, possible mechanisms for the antimicrobial effects of licorice root phytochemicals have been proposed [13]. More specifically, 18β-glycyrrhetinic acid decreases the expression of key virulence genes of methicillin-resistant *S. aureus* [14]; licochalcone A inhibits biofilm formation [15]; licochalcone E reduces the production of α-toxin [16]; liquiritigenin lowers the production of α-hemolysin; and both licochalcone A and glabridin prevent yeast–hyphal transition [15,17]. Accumulating evidence additionally suggests that *L. monocytogenes* is significantly susceptible to licorice root extracts and fractions [18–20]. Furthermore, the aqueous extract of licorice root demonstrates synergistic effects on the growth of *L. monocytogenes* with aminoglycosides such as gentamicin, with the presence of licorice extract reducing the minimum inhibitory concentration of gentamicin by 32-fold [21]. Previous works mainly focused on the anti-Listeria potency of glabridin, a prenylated isoflavan with well-known inhibitory effects against *L. monocytogenes*, with its bactericidal activity defined at concentrations above 25 $\mu g\ mL^{-1}$ [22,23].

Considering the growing interest in replacing synthetic food antimicrobials with natural ones, mainly because of the public's conviction that natural antimicrobials are safer than synthetic analogs, the food industry is strongly inspired to discover safe and low-cost antimicrobial agents of natural origin [24]. Initial work has shown that licorice root extract could inhibit the growth of a range of Gram-positive bacteria, including *L. monocytogenes* [25]. Therefore, the objective of the present work was to explore for the first time the potential of licorice root extract (LRE) obtained by supercritical fluid extraction to control the growth of different *Listeria* species, since outstanding differences in susceptibility among the Listeria species were found for natural and synthetic substances. Subsequently, the major and minor phytochemicals in LRE were elucidated with the

employment of LC-DAD-qTOF-MS in order to explain its antimicrobial effects. Finally, the AntiBac-Pred, a web application for predicting the antibacterial activity of chemical compounds, was used to pinpoint the active phytoconstituents of LRE.

2. Materials and Methods

2.1. Chemicals, Bacterial Strains and Extract

Listeria agar according to ISO 11,290 (Merck®, Darmstadt, Germany), brain–heart infusion (BHI) broth (Himedia®, Mumbai, India), and absolute ethanol (Scharlau Chemie, Barcelona, Spain) were used in microbiological experiments.

Quercetin and rutin standards were supplied by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile of HPLC grade and acetic acid were also purchased from Sigma-Aldrich, and they were used for the phytochemical analysis of the extract.

L. monocytogenes EGD (serotype 1/2a), *L. monocytogenes* Scott A (serotype 4b), *L. monocytogenes* NCTC 4885 (serotype 4b), *L. monocytogenes* NCTC 4994 (serotype 4b), *L. monocytogenes* ATCC 23,074 (serotype 4b), *L. monocytogenes* NCTC 1792 (serotype 4b), *L. monocytogenes* NCTC 7973 (serotype 1/2a), *L. grayi* NCTC 10815, *L. seeligeri* NCTC 11856, and *L. ivanovii* NCTC 11,846 were used in the present study.

LRE was prepared by the CO_2 extraction of dried *Glycyrrhiza uralensis* root and provided by Flavex Naturextrakte GmbH (Rehlingen, Germany). The extractor was filled with 70 kg of dried *Glycyrrhiza uralensis* root and was operated at 30 MPa and 40 °C using a CO_2 mass flow rate of 50 kg per kg of plant material.

2.2. Determination of Antimicrobial Activity by Broth Microdilution Method

The broth microdilution method was performed using the Tecan Infinite 200 PRO plate reader (Tecan Group, Männedorf, Switzerland) to determine the minimum inhibitory concentrations (MIC) of the LRE. Briefly, 50 μL of serial dilutions of LRE, ranging from 4 to 500 μg $mL^{-1,}$ were transferred, in triplicate, into a 96-well plate. Then, the extract was diluted with 40 μL of BHI broth and 10 μL of microbial suspension of a final concentration of 10^6 cfu mL^{-1} in each well. The inoculated 96-well plates were then incubated for 16 h at 37 °C. Optical density was measured and recorded every 30 min after shaking for 20 s [26]. The MIC was defined as the lowest concentration of extract that reduced the growth to an OD of 0.2 or less at the time of measurement.

2.3. Determination of Bactericidal Activity

The bactericidal activity of LRE was also determined using the Miles and Misra technique. Briefly, 50 μL of serial dilutions of LRE, ranging from 4 to 500 μg $mL^{-1,}$ were transferred, in triplicate, into a 96-well plate. Then, the extract was diluted with 40 μL of BHI broth and 10 μL of microbial suspension of a final concentration of 10^6 cfu mL^{-1} in each well. After incubation for 24 h at 37 °C, an appropriate volume from each well was taken, and 10-fold serial dilutions were prepared for each concentration. After that, an aliquot of 10 μL was placed in a BHI petri dish, which was previously divided into 6 parts representing the number of serial dilutions. Every part of the petri dish had 6 spots, representing 6 replicates [27]. The MBC was deemed to be the minimum concentration of extract capable of inactivating more than 99.99% of the bacteria present, resulting in no increase in optical density.

2.4. Identification and Quantification of Individual Phytochemicals in LRE

The LRE was further analyzed using UPLC/ESI-QTOF-MS to elucidate its phytochemical composition. The chromatographic separation was performed on an ACQUITY Ultra Performance LC system (Waters Corporation, Milford, MA, United States) equipped with a

vacuum degasser, an autosampler, a binary pump, and a DAD detector. A volume of 2 µL of diluted extract was loaded onto an RP18 column (1.7 µm, 2.1 mm × 100 mm; ACQUITY UPLC BEH Shield RP18) in the present chromatographic separation. The mobile phase was composed of acetic acid (1%, *v/v*) (Phase A) and acetonitrile (Phase B) and was pumped through the analytical column at a rate of 0.6 mL min^{-1}. The gradient elution program was set as follows: 0 min, 1% B; 2.3 min, 1% B; 4.4 min, 7% B; 8.1 min, 14% B; 12.2 min, 24% B; 16 min, 40% B; 18.3 min, 100% B, 21 min, 100% B; 22.4 min, 1% B; and finally, a conditioning cycle of 3 min with the initial conditions.

MS analyses were performed with the employment of a Q/TOF micro mass spectrometer (Waters Corporation, Milford, MA, USA). The negative ionization mode was used, and the conditions were as follows: cone gas flow, 40 L/h; drying gas flow (N_2), 11,000 L/h; nebulizer pressure, 50 psi; gas drying temperature, 360 °C; capillary voltage, 2500 V; fragmentor voltage and scan range, 3500 V and m/z 50–1500, respectively. The compounds were monitored at 280 nm. Integration and data elaboration were performed using MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). The quantification was performed using the calibration curves that were prepared from the limit of quantification (LOQ) to 100 µg mL^{-1} obtained by MS. All calibration curves showed good linearity among different concentrations ($r > 0.999$).

2.5. In Silico Screening of the Antimicrobial Potency of LRE

The major phytoconstituents of LRE, identified by LC-DAD-qTOF-MS, were included in the computational analysis. The two-dimensional structures and canonical simplified molecular input line entry system (SMILES) of the compounds were obtained from the PubChem Compound page at https://pubchem.ncbi.nlm.nih.gov/, accessed on 10 September 2024. Subsequently, the chemical structures were subjected on the AntiBac-Pred web tool of the Way2Drug platform (https://www.way2drug.com/antibac/), accessed on 10 September 2024, in order to predict their inhibitory effect on the growth of *Listeria monocytogenes* bacteria.

3. Results & Discussion

3.1. Evaluation of the Anti-Listerial Potency of LRE

The anti-Listerial potency of LRE was investigated by the employment of the broth microdilution method. Initially, the MIC of the licorice root extract was determined for each *Listeria* species and strain, as this value reflects the concentration required to inhibit visible bacterial growth and serves as an indicator of the extract's bacteriostatic effect against each test organism. LRE was obtained by supercritical carbon dioxide extraction; thus, water and ethanol were used for the solubilization of the extract for the assessment of its inhibitory effect against *Listeria* bacteria. Table 1 summarizes the MIC values of LRE dissolved in ethanol and water against *Listeria* strains. In particular, the MICs of LRE ranged from 31.3–65.5 µg mL^{-1} when dissolved in ethanol, whereas MICs were significantly higher when water was used for solubilization (125–250 µg mL^{-1}). The significant differences in MIC values may be attributed to the better solubilization of LRE phytochemicals in ethanol than water. The superiority of alcohol for the solubilization of phytochemicals against water has been described thoroughly [28]. Findings also support the great potential of LRE as a growth inhibitor of *Listeria* bacteria, since its MIC values were similar to well-known classes of antibiotics such as cephalosporins and quinolones and comparable with streptogramins and lincosamides [29]. The MIC values of LRE are also substantially lower than those of over a hundred plant materials (entire plants, bulbs, fruits, flowers, roots, seeds, and by-products) published from 2017 to 2023 [6]. The potential of LRE to inhibit the growth of *Listeria* bacteria is equivalent to extracts of *Hypericum perforatum* L.

leaves [30], *Berberis libanotica* Ehrenb. leaves [31], *Pistacia lentiscus* leaves [32], *Origanum ehrenbergii* Boiss aerial parts [33], and *Alpinia galanga* (Linn.) flowers [34]. In addition, LRE is more effective in controlling the growth of *Listeria* bacteria than extracts originating from the aerial parts and roots of *Glycyrrhiza glabra*. Its aerial parts and root extracts present MIC values of 620 μg mL^{-1} and 290 μg mL^{-1}, respectively [18,19]. Since bacteriostatic effects alone are insufficient to confer antiseptic and disinfectant properties on an extract or antibacterial agent, the bactericidal activity of LRE was also determined. According to Table 1, the MBC values of the extract dissolved in ethanol ranged from 62.5 μg mL^{-1} to 250 μg mL^{-1}. Interestingly, the variation of MBC values is greater than that of MICs. *L. monocytogenes* ATCC 23,074 and *L. monocytogenes* NCTC 7973 were the most susceptible strains among those studied. On the other hand, significantly higher amounts of LRE were required to kill the bacteria of *L. monocytogenes* EGD, *L. grayi* NCTC 10815, and *L. seeligeri* NCTC 11856. Results also highlighted that non-pathogenic *Listeria* species are more resistant to the effect of the extract in comparison with pathogenic *Listeria*. Results disclose remarkable differences in inherent susceptibility among the studied species and strains. The susceptibility diversity of *Listeria* species to antibiotics and natural products has also been described [29,35,36]. In addition, the MIC values reveal a great potential of LRE to act as a potent inhibitor of *Listeria* bacteria growth, since they meet stringent end point criteria for "activity" as a previous study described [37]. In particular, plant extracts should be considered efficacious if they exhibit IC_{50} values $\leq$ 100 μg mL^{-1}; the present extract is notably promising, as its MBC values are lower than the described critical concentration. Therefore, the elucidation of phytochemical composition is of great importance in order to decipher the antimicrobial potential of LRE.

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of licorice root extract dissolved in ethanol and water against *Listeria* strains. MIC and MBC values are expressed as (μg mL^{-1}).

***Listeria* Strains and Serotypes**	**MIC (μg mL^{-1})**		**MBC (μg mL^{-1})**	
	Ethanol	**Water**	**Ethanol**	**Water**
L. monocytogenes ATCC 23,074 (serotype 4b)	31.3	125	62.5	500
L. monocytogenes EGD (serotype 1/2a)	31.3	125	250	500
L. monocytogenes Scott A (serotype 4b)	62.5	250	125	500
L. monocytogenes NCTC 7973 (serotype 1/2a)	31.3	125	62.5	250
L. monocytogenes NCTC 4885 (serotype 4b)	62.5	125	125	250
L. monocytogenes NCTC 4994 (serotype 4b)	62.5	125	125	250
L. monocytogenes NCTC 1792 (serotype 4b)	62.5	125	125	500
L. ivanovii NCTC 11846	62.5	250	125	500
L. grayi NCTC 10815	62.5	250	250	500
L. seeligeri NCTC 11856	31.3	250	250	500

3.2. Elucidation of the Phytochemical Composition of LRE

The phytochemical composition of extracts is strongly linked to their antimicrobial potential. Previous works focused on glabridin, a prenylated flavonoid that could be extracted from licorice roots and has shown antimicrobial activity against *Listeria* bacteria and other foodborne pathogens and spoilage microorganisms [38,39]. Glabridin can reduce *L. monocytogenes* motility and hemolytic activity, but was not found to have antimicrobial activity [38,39]. Furthermore, prenylated (iso)flavonoids and chalcones from roots exert potent inhibitory effects on many bacteria and yeasts [40]. Thus, the phytochemical composition of LRE was elucidated using an advanced LC-DAD- qTOF-MS set-up to accurately identify its active phytoconstituents. Peak identification was based on retention times, UV–Vis spectra, mass spectra, and mass information from the literature. Chromatographic

analysis revealed an extract rich in phytochemicals with great structural diversity. Table 2 summarizes the identified compounds; the presence of all phytochemicals has been previously described in plants belonging to the *Glycyrrhiza* genus and their derivatives [41–45]. The majority of LRE phytochemicals belong to the flavonoid class, as previous studies reported [46,47]. More specifically, a great variety of flavanones, as well as flavones and chalcones, were found in the extract. In addition, LRE contains triterpenoids typical of licorice extracts, while the presence of coumarins and phenolic acid was also confirmed. The presence of all these compounds has been reported in licorice extracts in previously published works. Table 2 also clearly demonstrates that the liquiritin-apioside isomers are the major constituents of LRE, as their total content reaches 133.1 mg g^{-1} of extract. The results show a concentrated content of these isomers in LRE compared to licorice root extracts of different geographical origins [48]. [(2Z)-3-(4-tert-Butyl-2-hydroxyphenyl)-3-hydroxyprop-2-enoyl]benzoic acid was also found at high concentrations in LRE (32.9 ± 0.8 mg g^{-1} extract). Licorice glycoside isomers are also prominent constituents in LRE. These are glycosylated flavonoids, which are typical compounds in *Glycyrrhiza glabra* roots. In addition, LRE contains significant amounts of flavanone-type compounds: namely, kanzonol C, kanzonol W, and kanzonol Y. Unfortunately, the triterpenoid content of LRE could not be determined. According to previous works, the glycyrrhizin contents in *G. glabra* roots vary from 11 to 80 mg g^{-1}, depending on genetic and environmental factors, while glycyrrhetinic and oxo glycyrrhetinic acids, hydrolytic products of glycyrrhizin, are present in licorice root extracts at very low levels [49,50].

Table 2. Identification and quantification of phytoconstituents in licorice root extract using LC-DAD-qTOF-MS.

Peak	RT (min)	Molecular Formula	*m/z* Experimental [M-H]−	*m/z* Calculated [M-H]−	Score	Proposed Compound	Content (mg g^{-1})	Class	Reference
1	6.07	C27H30O15	593.1497	593.1506	99.92	vicenin 2 (apigenin-6,8-diC-glucoside)	1.2 ± 0.0	Flavone	[41]
2	7.44	C26H30O13	549.1610	549.1608	99.95	liquiritin-apioside (liquiritigenin-7-hexose-pentose)	43.0 ± 0.1	Flavanone	[41]
3	8.04	C26H30O13	549.1597	549.1608	98.83	liquiritin-apioside (liquiritigenin-7-hexose-pentose) isomer	39.8 ± 0.2	Flavanone	[41]
4	8.72	C26H30O14	565.1564	565.1557	96.6	butin 4′-O-(2″-O-ß-D-apiofuranosyl)-ß-D-glucopyranoside/Glycosidic flavanon *	0.2 ± 0.0	Flavanone	[51]
5	9.17	C33H38O18	721.1982	721.1980	99.52	3-Hydroxyl-3-methylglutaroyl-violanthin	0.5 ± 0.0	Flavone	[41]
6	9.34	C26H30O14	565.1522	565.1557	99.55	butin 4′-O-(2″-O-ß-D-apiofuranosyl)-ß-D-glucopyranoside/Glycosidic flavanon *	0.8 ± 0.2	Flavanone	[51]
7	9.76	C27H30O13	561.1596	561.1608	91.36	glycyroside (formononetin-7-hexose-pentose)	6.0 ± 0.2	Isoflavone	[41]
8	10.68	C15H12O4	255.0634	255.0657	-	Liquiritigenin	2.7 ± 0.0	Flavanone	[44]
9	11.11	C26H30O13	549.16	549.1608	96.6	iso/liquiritin-apioside (iso/liquiritigenin-7-hexosepentose)	50.3 ± 3.2	Flavanone	[41]
10	11.2	C21H22O9	417.1161	417.1186	95.14	Liquiritin; Neoisoliquiritin	1.1 ± 0.1	Flavanone	[41]
11	11.27	C16H14O5	285.0735	285.0763	99.44	LicoChalcone B	0.6 ± 0.0	Chalcone	[43]
12	11.631	C35H36O15	695.1982	695.1976	91.82	licorice glycoside D1/D2	nq *	Flavanoid	[43]
13	12.02	C36H38O16	725.2093	725.2082	92.81	licorice glycoside C1/C2	7.3 ± 0.3	Flavanoid	[43]

Table 2. *Cont.*

Peak	RT (min)	Molecular Formula	*m/z* Experimental [M-H]−	*m/z* Calculated [M-H]−	Score	Proposed Compound	Content (mg g^{-1})	Class	Reference
14	12.12	C35H36O15	695.1989	695.1976	94	licorice glycoside D1/D2	4.6 ± 0.3	Flavanoid	[41]
15	13.51	C36H38O16	725.2103	725.2082	98.69	licorice glycoside A	3.9 ± 0.3	Flavanoid	[41]
16	13.66	C35H36O15	695.1994	695.1976	99.75	licorice glycoside B	3.9 ± 0.2	Flavanoid	[41]
17	13.89	C16H12O4	267.0641	267.0657	97.47	Formononetin	3.8 ± 0.1	Isoflavone	[41]
18	15.29	C20H22O5	341.1369	341.1389	99.93	3′-prenyl-naringenin dihydrochalcone (3′-prenyl-2′,4′,6′,4-tetrahydroxydihydrochalcone)	3.8 ± 0.2	Chalcone	[41]
19	15.51	C20H22O5	341.1372	341.1389	99.13	3′-prenyl-naringenin dihydrochalcone (3′-prenyl-2′,4′,6′,4-tetrahydroxydihydrochalcone)	1.0 ± 0.0	Chalcone	[41]
20	15.689	C25H30O7	441.1915	441.1913	99.99	Norkurarinol	0.6 ± 0.1	Flavanone	[52]
21	15.76	C20H20O5	339.1232	339.1224	94.99	6(8)-prenylnaringenin	3.1 ± 0.1	Flavanone	[41]
22	15.88	C20H18O5	337.1076	337.1070	77.23 less	wighteone or lupiwighteone (8- or 6-prenylgenistein)	3.4 ± 0.0	Isoflavone	[41]
23	15.983	C25H30O7	441.192	441.1913	99.86	Norkurarinol	0.8 ± 0.0	Flavanone	[53]
24	16.095	C20H18O5	337.1076	337.1069	95.24	wighteone or lupiwighteone (8- or 6-prenylgenistein)	0.6 ± 0.1	Isoflavone	[41]
25	16.153	C42H62O16	821.399	821.396	94.49	Glycyrrhizin	nq	Saponin (triterpene glycoside)	[43]
26	16.211	C21H20O6	367.1182	367.1172	80.2	Gancaonin N/B/W	0.8 ± 0.0	Coumarin	[43]
27	16.44	C19H18O5	325.1066	325.1076	99.25	x-Hydroxymoracin N	5.3 ± 0.2	Coumarin	[43]
28	16.604	C21H20O5	351.123	351.1232	99.97	Gancaonin A/G/M, Glyurallin A	2.5 ± 0.1	Coumarin	[43]
29	16.649	C25H30O6	425.1964	425.1964	99.74	Kushenol T	2.5 ± 0.1	Flavanone	[43]
30	16.769	C25H30O6	425.1962	425.1964	98.54	Kushenol T	2.6 ± 0.1	Flavanone	[43]
31	16.815	C21H22O5	353.1389	353.1382	98.23	Licochalcone D	1.8 ± 0.0	Chalcone	[43]
32	16.894	C20H20O4	323.1283	323.1277	-	glabranin (8-prenylpinocembrin)	4.5 ± 0.0	Flavanone	[43]
33	16.931	C20H16O5	335.0919	335.0913	96.7	Kanzonol W; Licoagroisoflavone	5.4 ± 0.1	Isoflavone	[43]
34	16.993	C21H20O5	351.1232	351.1221	96.81	Gancaonin A/G/M, Glyurallin A	1.6 ± 0.1	Coumarin	[43]
35	17.055	C20H20O5	339.1232	339.1217	99.85	4-[(2Z)-3-(4-tert-Butyl-2-hydroxyphenyl)-3-hydroxyprop-2-enoyl]benzoic acid	32.9 ± 0.8	Benzoic acid	[43]
36	17.117	C20H18O6	353.1025	353.1013	89.23	Lico-iso-flavone A; Glycyrrh-iso-flavone; Allolicoisoflavone A	0.9 ± 0.0	Isoflavone	[43]
37	17.142	C25H30O6	425.1959	425.1964	99.23	Kushenol T	2.5 ± 0.1	Flavanone	[43]
38	17.171	C21H22O5	353.1378	353.1389	98.32	Licochalcone D	1.0 ± 0.1	Chalcone	[43]
39	17.22	C21H20O6	367.1182	367.1172	88.72	Gancaonin N/B/W	2.7 ± 0.1	Coumarin	[43]
40	17.295	C25H30O5	409.2015	409.201	96.62	Kanzonol Y	3.2 ± 0.1	Isoflavone	[43]
41	17.36	C21H20O5	351.1214	351.1232	91.61	Gancaonin A/G/M, Glyurallin A	2.2 ± 0.1	Coumarin	[43]
42	17.365	C25H30O5	409.2005	409.201	99.81	Kanzonol Y	4.2 ± 0.2	Isoflavone	[43]
43	17.415	C25H28O4	391.109	391.1885	100	kanzonol C/Hispaglabridin A	0.4 ± 0.0	Isoflavone	[43]
44	17.45	C21H22O4	337.1411	337.144	99.06	Licochalcone A/C/E	0.8 ± 0.1	Chalcone	[43]

Table 2. *Cont.*

Peak	RT (min)	Molecular Formula	*m/z* Experimental [M-H]−	*m/z* Calculated [M-H]−	Score	Proposed Compound	Content (mg g^{-1})	Class	Reference
45	17.576	C30H46O4	469.3299	469.3318	99.97	Glycyrrhetinic acid	nq	Triterpenoid	[41]
46	17.638	C25H26O5	405.1677	405.1702	90.63	Gancaonin Q/Glycyrdione B	1.7 ± 0.0	Coumarin	[43]
47	17.669	C30H44O4	467.3144	467.3161	91.28	Oxoglycyrrhetinic acid	nq	Triterpenoid	[43]
48	17.7	C25H28O4	391.1893	391.109	99.96	kanzonol C/Hispaglabridin A	0.1 ± 0.0	Isoflavone	[43]
49	17.812	C25H28O5	407.1858	407.1845	99.77	Glyinflanin A/Hydroxyglabrol	2.6 ± 0.1	Chalcone	[44]
50	17.899	C40H36O10	675.2217	675.223	99.91	Guangsangon F	0.3 ± 0.0	Flavanone	[43]
51	17.99	C25H28O4	391.1909	391.1884	99.8	Glabrol/kanzonol C/Hispaglabridin A	0.4 ± 0.0	Isoflavone	[43]

* nq: not quantified.

3.3. In Silico Evaluation of the Antibacterial Potency of LRE Phytoconstituents

Computational tools are widely used for the prediction of the antimicrobial activity of diverse groups of antimicrobials. The most common target predictions are based on individual molecules. Ligand-based methods include methods that are based on the principle of chemical similarity, which states that similar chemical structures tend to present similar antimicrobial activities more often than not, although even structurally similar compounds may interact with a protein target in different ways [54,55]. In this study, 51 phytochemicals were identified in LRE. The computational screening was applied to phytochemicals with content above 0.5% *w*/*w*, as well as to triterpenoids, whose content was not calculated for technical reasons. The application of computation tools is expected to pinpoint the antimicrobial components of extracts, avoiding laborious and time-consuming fractionation and purification procedures. Thus, the antibacterial activities of LRE phytochemicals against *Listeria monocytogenes* were determined using the online tool AntiBac-Pred of the Way2Drug platform. Antibac-Pred analyzes growth inhibitors or non-inhibitors of *L. monocytogenes* based on antibacterial activity data available in ChEMBL, a database of bioactive molecules with drug-like properties (Figure 1). The score for each compound is expressed as confidence in its activity, which represents the difference between the probabilities that a chemical compound inhibits or does not inhibit the growth of a given bacteria. As confidence increases, the chances of the prediction being true are greater [56]. The calculated confidence scores (Table 3) show that the most promising inhibitor of *L. monocytogenes* is liquiritin-apioside, followed by licorice glycoside C1/C2. Interestingly, similar confidence scores were calculated for well-established antibiotics such as ampicillin and gentamicin. There is no data yet for the antibacterial potency of both pure phytochemicals. Isoliquiritin-apioside, glycyroside, and kanzonol W also appear to have the potential to act as possible anti-Listeria agents. Surprisingly, the typical triterpenoids of licorice roots, namely, glycyrrhizin and glycyrrhetinic acid, do not exert inhibitory effects against *Listeria* according to the confidence scores. However, previous works demonstrate their strong antimicrobial effects against bacteria such as *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi* [51–53,57,58]. The low confidence scores do not preclude the potent activity of compounds, since their structures are not typical of the "active" structures of the training set. Overall, the computational screening revealed the presence of many compounds with anti-Listerial potential that act in an additive or synergistic way. In addition, further studies are required to investigate the potency of LRE triterpenoids as inhibitors of *Listeria* bacteria.

Table 3. Prediction of inhibitory effects of licorice root extract phytoconstituents against *Listeria monocytogenes* via Antibac-Pred web tool.

Compound	PubChem CID	Smiles	Confidence Score
Liquiritin-apioside	10076238	C1[C@H](OC2 = C(C1 = O)C = CC(= C2)O)C3 = CC = C(C = C3)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O[C@H]5[C@@H]([C@](CO5)(CO)O)O	0.536
Isoliquiritin-apioside	6442433	C1[C@@]([C@H]([C@@H](O1)O[C@@H]2[C@H]([C@@H]([C@H](O[C@H]2OC3 = CC = C(C = C3)/C = C/C(= O)C4 = C(C = C(C = C4)O)O)CO)O)O)O)(CO)O	0.338
4-[(2Z)-3-(4-tert-Butyl-2-hydroxyphenyl)-3-hydroxyprop-2-enoyl]benzoic acid	6440678	CC(C)(C)C1 = CC(= CC(= C1O)C(C)(C)C)/C = C/C(= O)C2 = CC = C(C = C2)C(= O)O	-
Licorice glycoside C1/C2	42607809/101938907	COC1 = C(C = CC(= C1)/C = C/C(= O)OCC2(CO[C@H]([C@H]2O)OC3[C@H]([C@@H](C(O[C@H]3OC4 = CC = C(C = C4)[C@H]5CC(= O)C6 = C(O5)C = C(C = C6)O)CO)O)O)O)O	0.483
Glycyroside	101939210	COC1 = CC = C(C = C1)C2 = COC3 = C(C2 = O)C = CC(= C3)O[C@H]4[C@@H]([C@H]([C@@H]([C@H](O4)CO)O)O)O[C@H]5[C@@H]([C@](CO5)(CO)O)O	0.385
Kanzonol W	15380912	CC1(C = CC2 = C(O1)C = CC3 = C2OC(= O)C(= C3)C4 = C(C = C(C = C4)O)O)C	0.259
Glycyrrhizin	14982	C[C@]12CC[C@](C[C@H]1C3 = CC(= O)[C@@H]4[C@]5(CC[C@@H](C([C@@H]5CC[C@]4([C@@]3(CC2)C)C)(C)C)O[C@@H]6[C@@H]([C@H]([C@@H]([C@H](O6)C(= O)O)O)O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)C(= O)O)O)O)O)C)(C)C(= O)O	-
Glycyrrhetinic acid	10114	C[C@]12CC[C@](C[C@H]1C3 = CC(= O)[C@@H]4[C@]5(CC[C@@H](C([C@@H]5CC[C@]4([C@@]3(CC2)C)C)(C)C)O)C)(C)C(= O)O	-
Ampicillin	6249	CC1([C@@H](N2[C@H](S1)[C@@H](C2 = O)NC(= O)[C@@H](C3 = CC = CC = C3)N)C(= O)O)C	0.641
Gentamicin	3467	CC(C1CCC(C(O1)OC2C(CC(C(C2O)OC3C(C(C(CO3)(C)O)NC)O)N)N)N)NC	0.586

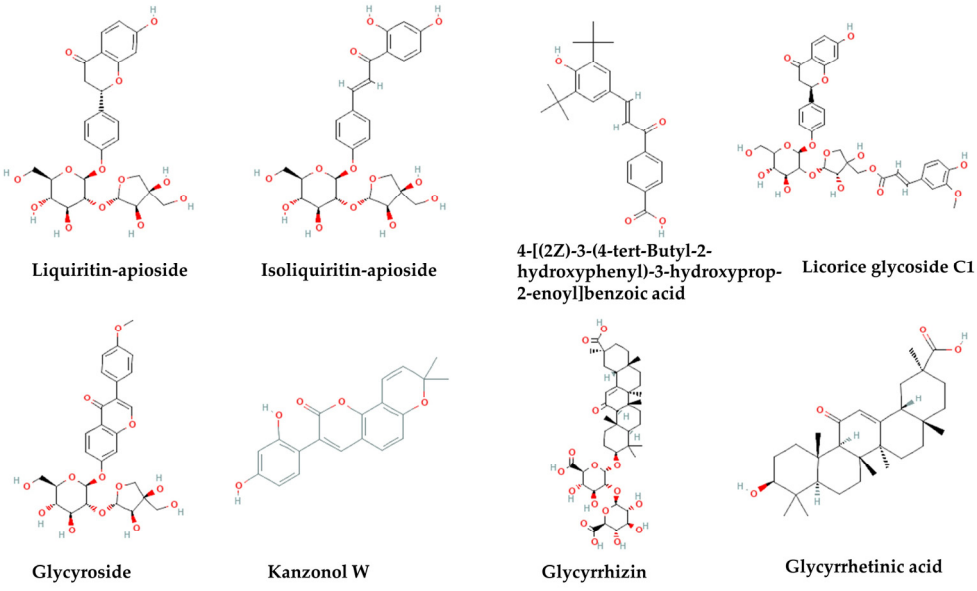

Figure 1. Structures of phytochemicals tested as *Listeria monocytogenes* inhibitors via Antibac-Pred web tool.

4. Conclusions

Overall, the results obtained herein suggest the ability of licorice root extract to control a panel of *Listeria* strains, a particularly important causative agent responsible for serious diseases in both humans and animals. In addition, the MIC and MBC values of this extract meet the endpoint criteria for "activity" as proposed in a previous critical review. LC-DAD-qTOF-MS analysis also demonstrated an extract rich in flavanones, flavones, chalcones, and triterpenoids, with their contribution to the antimicrobial effects of the extract having been studied. This work reveals the active components of the extract and offers new lead structures for controlling *Listeria* growth. Based on these findings, further fractionation and testing of the pure phytochemicals to assess their MIC and MBC values are strongly recommended. In conclusion, the licorice root can be included in the list of natural antimicrobial agents with potential uses in the food industry. However, more research is needed to fully understand the efficacy, safety, and regulatory aspects of using licorice in food preservation on a commercial scale.

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