Antibacterial activity of phenolic compounds derived from *Ginkgo biloba*sarcotestas against food-borne pathogens

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ABSTRACT

Aims: We investigated the antibacterial activity of three groups of phenolic compounds (1-4, 5-7, 8-10) obtained from an $CHCl_3$ extract of *Ginkgo biloba* L. (Ginkgoaceae) sarcotestas.

Study design: An experimental study.

Methodology: Inhibition of microbial growth was measured by an agar diffusion method and susceptibility tests were performed by the broth microdilution method.Bactericidal effect of *Ginkgo biloba* compound 5-7 against *Salmonella enterica*serovarTyphimurium was assessed by time-kill assay.

Results:*Ginkgo biloba* compounds 5-7 and 8-10 showed high antimicrobial activity against Gram-positive and Gram-negative bacteria, including several food-borne pathogens. In particular, compounds 5-7 and 8-10, containing phenolic acids and bilobols, respectively, were highly effective against *Salmonella enterica*serovarTyphimurium, *Listeria monocytogenes*, *Listeria innocua*, *Streptococcus pyogenes*, *Escherichia coli*, and *Shigelladysenteriae*. On the opposite, compounds 1-4, containing cardanols, showed little antibacterial activity. Compounds 5-7 exerted a bactericidal and bacteriolytic effect on *Salmonella enterica*serovarTyphimuriumwith a Minimal Inhibitory Concentration (MIC) and a Minimal Bactericidal Concentration (MBC) of 8.3 μg ml^{-1}.

Conclusion: The results of this study indicate that phenolic compounds derived from *Ginkgo biloba*sarcotestas, because of their strong inhibitory characteristics towards food pathogens, can be considered ideal candidates for possible application in food microbiology due to their natural origins.

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Keywords: Ginkgo biloba compounds; antibacterial activity; Salmonella entericaserovarTyphimurium; natural antimicrobials; food microbiology; food-borne pathogens

1. INTRODUCTION

Ginkgo biloba L. is a plant belonging to the Ginkgoaceae family and its beneficial effects on human health are largely known. In fact, Ginkgo has been used therapeutically for centuries in Traditional Chinese Medicine [1]. Currently, it represents one of the top-selling herbs in health food stores in the United States [2]. Among several compounds, *Ginkgo biloba* contains flavonoids (ginkgo-flavone glycosides) and terpenoids (ginkgolides and bilobalides).Its traditional preparations are used to ameliorate peripheral vascular disease, such as intermittent claudication and cerebral deficiency. Moreover, the ginkgolides inhibit platelet aggregation and the initial symptoms of arteriosclerosis [3-4].

In Western medicine, dry extracts of *Ginkgo biloba* leaves, known as Egb761 [3]are reported to improve mood and cognitive performance, to protect memory deficits and central nervous system disorders, to alleviate symptoms of mild/moderate Alzheimer-type dementia, to possess antidepressant and antioxidant properties [5-8]. Moreover, increasing evidence suggest a beneficial use of Egb in treating cardiovascular diseases [9-10].

Despite the numerous works on the healthy properties of *Ginkgo biloba*, little is known about its antimicrobial activity [11-17].

The aim of this study is to evaluate the *in vitro* antibacterial activity of three *Ginkgo biloba*sarcotestas-derived mixture of compounds, named 1-4, 5-7, and 8-10. These compounds, previously characterized as cardanols, phenolic acids, and bilobols, respectively [18] (Fig. 1), have been screened for antimicrobial activity against 11 bacterial strains, including Gram-positive, Gram-negative, pathogenic and safe strains. For mixtures 5-7 and 8-10, the minimal inhibitory and bactericidal concentrations were determined. Finally, we analyzed the kinetics of *Salmonella enterica*serovarTyphimurium growth inhibition by compounds 5-7.

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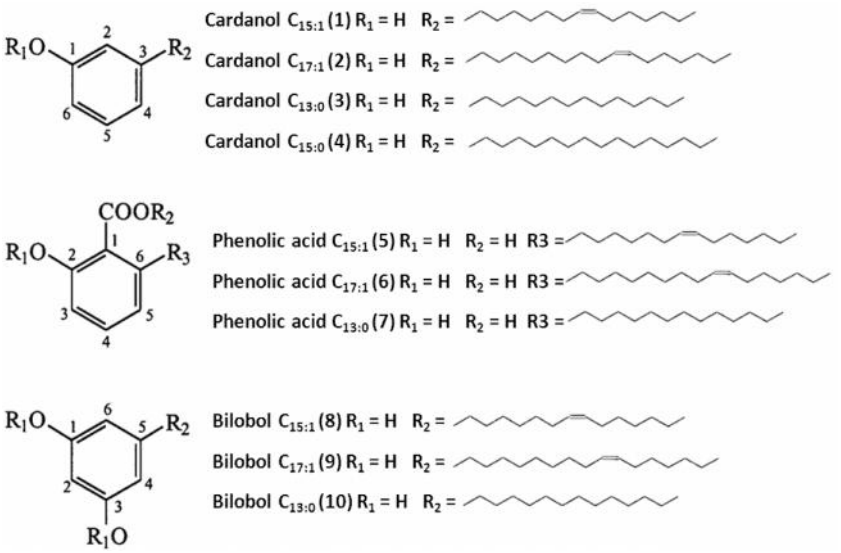

Fig. 1.Structures of *Ginkgo biloba* compounds. Adapted from Lee et al. (1998)

2. MATERIAL AND METHODS

2.1 Bacterial strains and media

Bacterial strains used in the present study were from the author's Institute collection or from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). *Escherichia coli, Enterobacteraerogenes, Pseudomonas aeruginosa, Salmonella enterica*serovarTyphimurium, *Shigelladysenteriae* ATCC 11835, *Staphylococcus aureus, Streptococcus pyogenes, Vibrio mediterranei,* and *Vibrio vulnificus* were grown in Nutrient Broth (NB, Becton Dickinson, Cockeysville, USA). *Listeria innocua* and *Listeria monocytogenes* were propagated in Tryptone Soya Broth (TSB, Oxoid Limited, Basingstoke, UK). The plating medium for the activity assay was Tryptone Soya Agar (TSA) containing 3% TSB and 1,5% agar (Oxoid).For *Vibrio mediterranei* and *Vibrio vulnificus*, each medium was supplemented with 3% NaCl according to the halophilic features of the strains.
For the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) determination, Mueller-Hinton II broth (BBL Microbiology Systems, Cockeysville, Md, USA) and TSA were used, respectively.

2.2 *Ginkgo biloba* compounds

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The fresh sarcotestas of *Ginkgobiloba* L. (Ginkgoaceae) were collected from the ginkgotrees in Korea and identifiedby Dr. Dae Suk Han, emeritus professor, Collegeof Pharmacy, Seoul National University, as previously reported [18]. The fresh sarcotestas were extractedin $CHCl_3$and purified by chromatographic methods exactly as described [18]. The structures of compounds 1-10 shown in Fig. 1 were identifiedby IR, ^{1}H NMR, ^{13}C NMR, and MS and confirmed by comparison with those ofliterature data [18 and references therein].The 10 phenolic compounds (Fig. 1) ~~from the $CHCl_3$ extract of *Ginkgo biloba* L. sarcotestas (Ginkgoaceae) were isolated and, based on their chemical structures,~~were pooled in three groups: cardanols (compounds 1-4), phenolic acids (compounds 5-7), and bilobols (compounds 8-10)(Fig. 1). For the present study, all these compounds were dissolved in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany), and kept in aliquots at –20°C, before use in antimicrobial tests.

2.3 Antibacterial assay

We used an agar diffusion method [19]. Briefly, suspensions of bacterial strains grown overnight in appropriate media were adjusted to 10^8CFU ml^{-1}, inoculated in 8 ml of molten Tryptone Soya Agar (0.7% agar) and poured over TSA plates (agar 1.5%). After cooling and drying, 10 μl of each *Ginkgo biloba*mixture,e.g., 1-4, 5-7, 8-10, corresponding to 33,3 μg, were applied as a spot and allowed to diffuse. The plates were then inverted and incubated for 18-24 h at the optimal temperature of the test organism. The presence of a clear zone around the spot indicated growth inhibition and the diameter of the zone of inhibition was measured. As a positive control, a mixture of the following antibiotics was used: Pen/Strep (penicillin 5000 IU/ml; streptomycin 5000 μg ml^{-1} (Life Technologies, Milan, Italy). DMSO and Nutrient broth were employed as negative controls. In a different experiment, serial dilutions of *Ginkgo biloba*mixtures 1-4, 5-7, 8-10 were spotted as above to verify the dose-dependence of the inhibition in solid media. The highest concentration applied was 3330 μg ml^{-1}.

2.4 Susceptibility testing

The in vitro activities of the *Ginkgo biloba* compounds against the most sensitive bacteria (*Salmonella enterica*serovarTyphimurium*, Listeria monocytogenes, Listeria innocua, Streptococcus pyogenes, Escherichia coli, Shigelladysenteriae* ATCC 11835) were determined by the EUCAST (European Committee on Antimicrobial Susceptibility Testing)broth microdilution method [20]. Briefly, microtiter plates containing serial 10-fold dilutions of each *Ginkgo biloba* compound, with concentration range varying from 3.33 to 3330 μg ml^{-1}, were inoculated in the presence of single strains

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to yield the appropriate density (10^5 CFU/ml). The plates were incubated aerobically for 24 h at 37°C. For a more accurate measurement of the MIC, the lowest concentrations of the compounds with activity underwent further two-fold dilutions. The wells without any visible growth of microorganisms were sub-cultured in order to determine the MBC, that was defined as the lowest concentration of the test compound that killed all the bacteria.

2.5 Bactericidal effect of *Ginkgo biloba* compound 5-7 against *Salmonella enterica*serovarTyphimurium

NB containing either 40 or 80 μl (corresponding to 133.2 and 266.4 μg, respectively) of *Ginkgo biloba* compounds 5-7 was inoculated in an overnight culture of *Salmonella enterica*serovarTyphimurium, grown at 37 °C in NB adjusted to $O.D_{590}$ = 0.1. Growth of *Salmonella enterica*serovarTyphimurium was monitored by measuring the optical density of broth cultures at 590 nm at various times, up to 24 h. Viable bacterial cells were enumerated by standard plate count method. Medium plus bacterial culture, and medium plus bacterial culture containing 80 μl DMSO were tested as controls.

3. RESULTS AND DISCUSSION

The spectrum of activity of *Ginkgo biloba* compounds was very broad. High levels of inhibitory activity were detected against *Salmonella enterica*serovarTyphimurium, *Listeria monocytogenes, Listeria innocua, Streptococcus pyogenes, Escherichia coli, Shigelladysenteriae*, while a minor activity was evidenced against *Enterobacteraerogenes, Vibrio vulnificus, Staphylococcus aureus*. Finally, *Pseudomonas aeruginosa*and *Vibrio mediterranei*were not affected (Table 1). Compounds 5-7 and 8-10 showed the highest efficacy, while compound 1-4 had little antimicrobial activity. In particular, *Salmonella enterica*serovarTyphimurium and *Listeria* spp. showed the highest sensitivity to *Ginkgo biloba* compounds.

Table 1. Antibacterial activity of *Ginkgo biloba*compounds expressed as diameter of inhibition zone (mm)

	Diameter of inhibition zone (mm)*		
Organisms	**Compounds 1-4****	**Compounds 5-7****	**Compounds 8-10****
Escherichia coli	4.1 ± 0.3	21.4 ± 0.7	17.0 ± 0.6
Enterobacteraerogenes	4.3 ± 0.2	10.8 ± 0.4	8.8 ± 0.6
Listeria innocua	5.5 ± 0.4	22.2 ± 0.9	20.2 ± 0.8
Listeria monocytogenes	4.7±0.2	24.0 ± 0.8	21.4 ± 0.8
Pseudomonas aeruginosa	ND	ND	ND

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*Salmonella enterica*serovarTyphimurium	6.0 ± 0.4	24.5 ± 0.8	24.4 ± 0.7
Shigelladysenteriae	3.7 ± 0.4	16.0 ± 0.8	15.5 ± 0.6
Staphylococcus aureus	4.6 ± 0.2	8.7 ± 0.7	8.3 ± 0.7
Streptococcus pyogenes	4.0 ± 0.0	21.5 ± 0.6	17.5 ± 0.3
Vibrio mediterranei	ND	ND	ND
Vibrio vulnificus	3.5 ± 0.1	10.5 ± 0.4	8.8 ± 0.7

*The values are expressed as mean ± standard deviation (SD) of three independent experiments.
ND, not determined (zone of inhibition was no greater than 3 mm).
**Concentration applied: 3330 μg ml^{-1}.

Analyzing the diameter of the inhibition zone (Table 1) and the results of susceptibility test (Table 2), mixture 5-7 was slightly more active than 8-10 against all strains tested. In fact, MIC and MBC values were in the range of 8.3-33.3 μg ml^{-1} and 8.3-333.0 μg ml^{-1}, respectively, for mixture 5-7, while the same parameters ranged between 8.3-33.3 μg ml^{-1} and 16.6-333.0 μg ml^{-1} for compounds 8-10. More in details, compounds 5-7 was strongly inhibitory against *Salmonella enterica*serovarTyphimurium (MIC and MBC: 8.3 μg ml^{-1}) and *Listeriamonocytogenes* (MIC and MBC: 8.3 μg ml^{-1} and 16.6 μg ml^{-1}, respectively).

Table 2. MIC and MBC values for *Ginkgo biloba*compounds 5-7 and 8-10 expressed as μg ml^{-1}

	Compound 5-7		**Compound 8-10**	
Organisms	**MIC**	**MBC**	**MIC**	**MBC**
Escherichia coli	16.6	33.3	33.3	333.0
Listeria innocua	16.6	16.6	16.6	33.3
Listeria monocytogenes	8.3	16.6	16.6	33.3
*Salmonella enterica*serovarTyphimurium	8.3	8.3	8.3	16.6
Shigelladysenteriae	33.3	333.0	33.3	333.0
Streptococcus pyogenes	16.6	33.3	33.3	333.0

The analysis of growth inhibitionkinetic triggered by compounds 5-7 against *Salmonella enterica*serovarTyphimurium indicated a bactericidal effect, as resulted by cell lysis (data not shown) and loss of turbidity in the growth medium (Fig. 2). Growth inhibition was detected starting from 2 h with a gradual increase over time. Both growth rate and cell density in treated

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cultures decreased doubling the amount of compounds 5-7 added to the culture (Fig. 2). DMSO, used as negative control, did not inhibit *Salmonella enterica*serovarTyphimuriumgrowth.

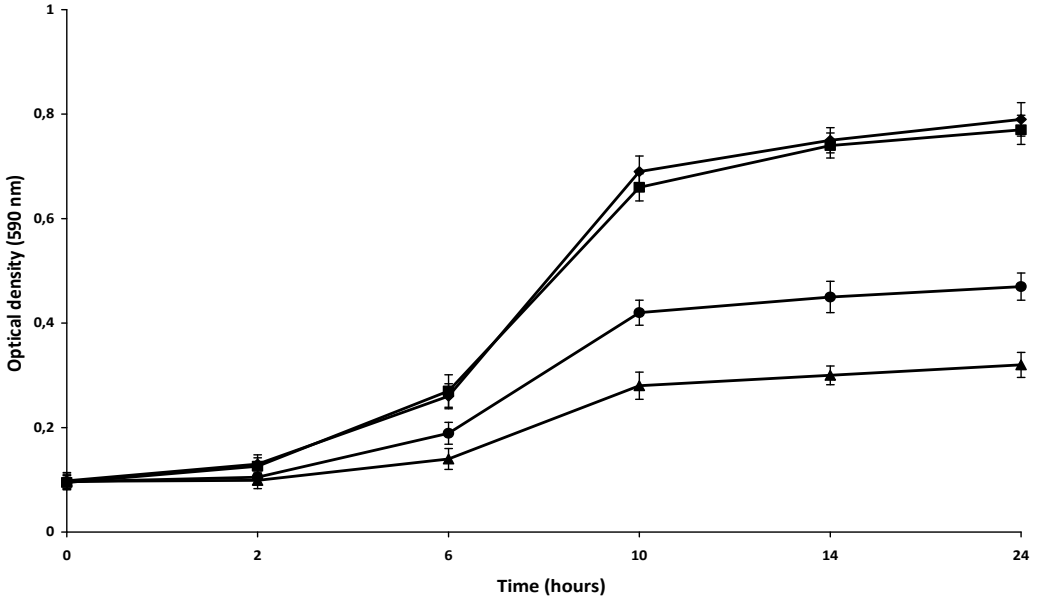

Fig.2.Inhibitory effect of *Ginkgo biloba* mixture 5-7 on *Salmonella enterica*serovarTyphimurium growth.*Ginkgo biloba* mixture 5-7, 40 µl (●) or 80 µl (▲)corresponding to 133.2 and 266.4 µg, respectively, was added to an early log-phase culture of *Salmonella enterica*serovarTyphimurium. Optical density (OD) at 590 nm was measured. Controls were medium plus bacterial culture without compound 5-7 (♦), and medium plus bacterial culture containing 80 µl DMSO (■). Values represent mean of three separate experiments ± standard deviation (SD).

In Fig. 3, it is reported the dose-effect of the three compounds against *Salmonella enterica*serovarTyphimurium in solid media. The linear correlation between volume of inoculum and size of inhibition halos is evident. The presence of a clear zone around the spotted area confirmed the bactericidal mode of action of compounds 5-7.

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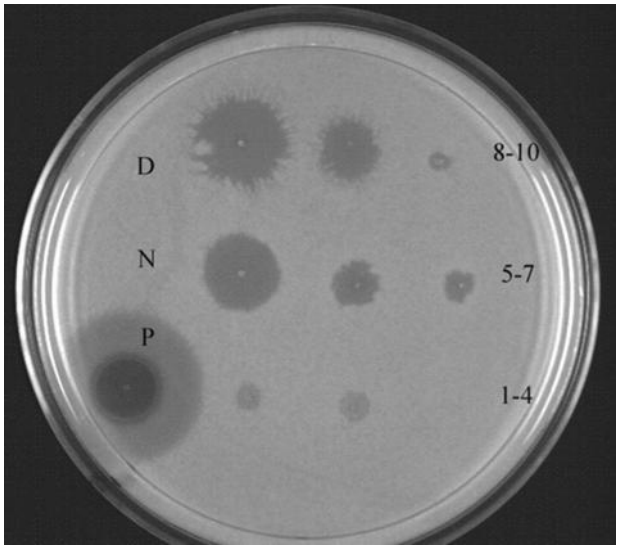

Fig.3. **Antibacterial activity of *Ginkgo biloba* compounds.***Salmonella enterica*serovarTyphimurium (10^8CFU ml^{-1}) was inoculated into 8 ml of molten TSA, and poured over TSA plates. Serial dilutions of compounds 8-10, 5-7, and 1-4 were spotted on the plate. Controls were DMSO (D), Nutrient Broth (N), and Pen/Strep (P).

Salmonellosis continues to be a major public health problem worldwide and *Salmonellaenterica*serovarTyphimurium is one of the most prevalent serovars among *Salmonella* spp. causing gastroenteritis [21]. Rates of multidrug-resistance in *Salmonella* spp. increased considerably in recent years, primarily in response to antimicrobial usage in humans and food animals. An example is the global spread of multidrug-resistant *Salmonella enterica*serovarTyphimurium phage type DT104, resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines[22]. *Listeria monocytogenes*is an important pathogen which has been isolated from various foodstuffs such as meat, poultry, eggs, seafood, and represents a major concern to food manufacturers [23]. In recent years, there has been an increasing interest in the development of effective natural antimicrobials as food preservatives. *Ginkgo biloba*compounds used in this study showed antibacterial activity against a wide panel of bacteria, including most of the tested pathogens.

Previous studies regarding the antimicrobial activity of *Ginkgo biloba* extracts showed contrasting results. In fact, Mazzanti*et al*. [12] reported that the antimicrobial activity of three fractions of methanolic extracts of *Ginkgo biloba* leaves was effective towards Gram-positive bacteria only.Lee and Kim [13] found that *Ginkgo biloba* leaf-derived materials inhibited *Clostridium perfringens* and *Escherichia coli*, but did not inhibit intestinal bacteria, such as *Bifidobacteriumbifidum* or *Lactobacillus acidophilus*. Boonkaew and Camper [14] reported that methanolic extracts from leaf and root tissue of

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Ginkgo biloba showed no inhibitory activity, but extracts from leaf and root derived callus inhibited the growth of *Klebsiellapneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus* spp., and *Streptococcus pyogenes*. No activity against *Escherichia coli* was detected. Sawano*et al*. [15] isolated a protein from the seeds of *Ginkgo biloba* that inhibited the growth of some fungi, but did not exhibit antibacterial activity against *Escherichia coli*. Sati and Joshi [16] showed that the methanolic extract of *Ginkgo biloba*leaves possessed inhibitory activity against *Escherichia coli*, *Bacillus subtilis* and some plant pathogenic bacterial strains. Finally,Tao *et al.*[17] reported the antibacterial/antifungal activities and synergistic interactions between *Ginkgo biloba*polyprenols and eight compounds separated from *Ginkgo biloba* L. leaves lipids against *Salmonella enterica*, *Staphylococcocusaureus* and *Aspergillusniger*.

The results of the present study indicate that *Ginkgo biloba*sarcotestas-derived compounds possess a remarkably high inhibitory activity against a wide spectrum of Gram-positive and Gram-negative bacteria. It is worthwhile to note that the tested compounds were highly active against *Salmonella enterica*serovarTyphimuriumand *Listeriamonocytogenes*. Moreover, *Ginkgo biloba*compounds 5-7 and 8-10 were also inhibitory towards other important food-borne pathogens, such as *Shigelladysenteriae*, *Escherichia coli* and the human pathogens *Staphylococcus aureus*and *Streptococcus pyogenes*.

4. CONCLUSION

Increase in antibiotic resistance, including multiple antibiotic resistance among several groups of bacteria, is a global phenomenon [24-25]. Reports on resistance in *Listeria* spp. have been previously published [26-28]. Therefore, new antimicrobial substances to counteract antibiotic resistance is urgently need. *Ginkgo biloba*mixtures 5-7 and 8-10 may represent ideal, natural candidates in potential applications aimed to preserve microbiological contamination of foods.

Further studies are needed to evaluate the antimicrobial activity of the individual components of the *Ginkgo biloba* compounds used in the present study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors AC and GLR designed the study, wrote the protocol and the draft of the

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manuscript. Authors AC, KR, and IT managed the analyses of the study and literature searches. Author JK prepared all the extracts of *Ginkgo biloba*. All authors read and approved the final manuscript.

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