Research Paper

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

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* serovar Typhimurium 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* serovar Typhimurium, *Listeria monocytogenes*, *Listeria innocua*, *Streptococcus pyogenes*, *Escherichia coli*, and *Shigella dysenteriae*. On the opposite, compounds 1-4, containing cardanols, showed little antibacterial activity. Compounds 5-7 exerted a bactericidal and bacteriolytic effect on *Salmonella enterica* serovar Typhimurium with 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.

Keywords: *Ginkgo biloba compounds; antibacterial activity; Salmonella enterica serovar Typhimurium; 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 [11-16].

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 [17] (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* serovar Typhimurium growth inhibition by compounds 5-7.

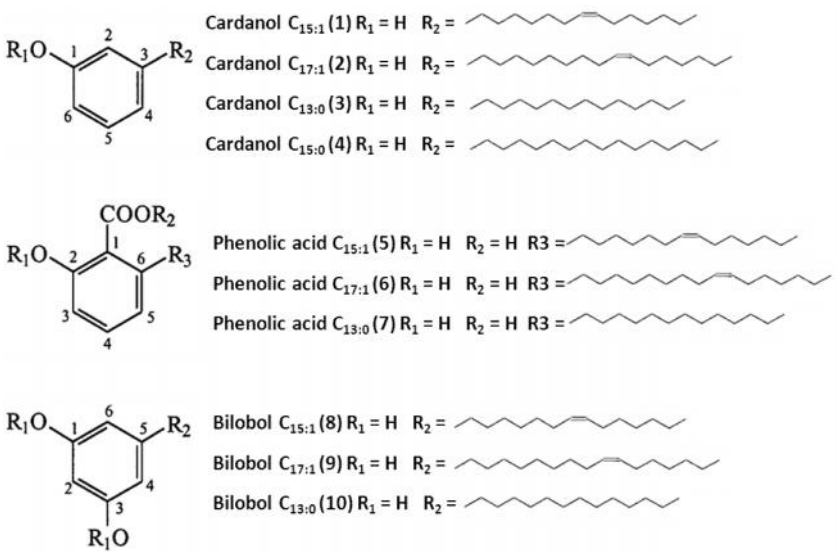

Fig. 1. Structures of *Ginkgo biloba* compounds. Adapted from Lee et al. (1998)

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, Enterobacter aerogenes, Pseudomonas aeruginosa, Salmonella enterica* serovar Typhimurium, *Shigella dysenteriae* 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

As previously reported [17], 10 phenolic compounds 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 [18]. Briefly, suspensions of bacterial strains grown overnight in appropriate media were adjusted to 10^8 CFU 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 was employed as negative control. 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* serovar Typhimurium*, Listeria monocytogenes, Listeria innocua, Streptococcus pyogenes, Escherichia coli, Shigella dysenteriae* ATCC 11835) were determined by the broth microdilution method [19]. 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 to yield the appropriate density (10^5 CFU/ml). The plates were incubated aerobically for 24 h at 37°. 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* serovar Typhimurium

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* serovar Typhimurium, grown at 37 °C in NB adjusted to $O.D_{590}$ = 0.1. Growth of *Salmonella enterica* serovar Typhimurium 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* serovar Typhimurium, *Listeria monocytogenes, Listeria innocua, Streptococcus pyogenes, Escherichia coli, Shigella dysenteriae*, while a minor activity was evidenced against *Enterobacter aerogenes, 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* serovar Typhimurium 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
Enterobacter aerogenes	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
Salmonella enterica serovar Typhimurium	6.0 ± 0.4	24.5 ± 0.8	24.4 ± 0.7
Shigella dysenteriae	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 the 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* serovar Typhimurium (MIC and MBC: 8.3 μg ml^{-1}) and *Listeria monocytogenes* (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 serovar Typhimurium	8.3	8.3	8.3	16.6
Shigella dysenteriae	33.3	333.0	33.3	333.0
Streptococcus pyogenes	16.6	33.3	33.3	333.0

The analysis of growth inhibition kinetic triggered by compounds 5-7 against *Salmonella enterica* serovar Typhimurium 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 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* serovar Typhimurium growth.

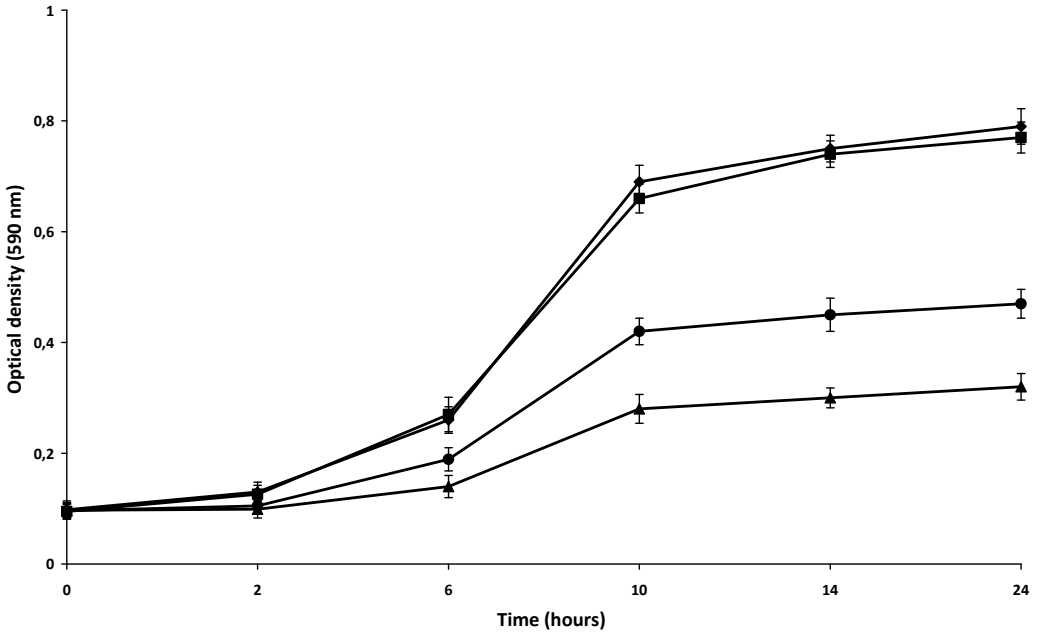

Fig. 2. Inhibitory effect of *Ginkgo biloba* mixture 5-7 on *Salmonella enterica* serovar Typhimurium growth. *Ginkgo biloba* mixture 5-7, 40 µl (●) or 80 µl (▲) corresponding to 133.2 and 266.4 µg, respectively, was added to an early log-phase culture of *Salmonella enterica* serovar Typhimurium. Optical 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* serovar Typhimurium 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.

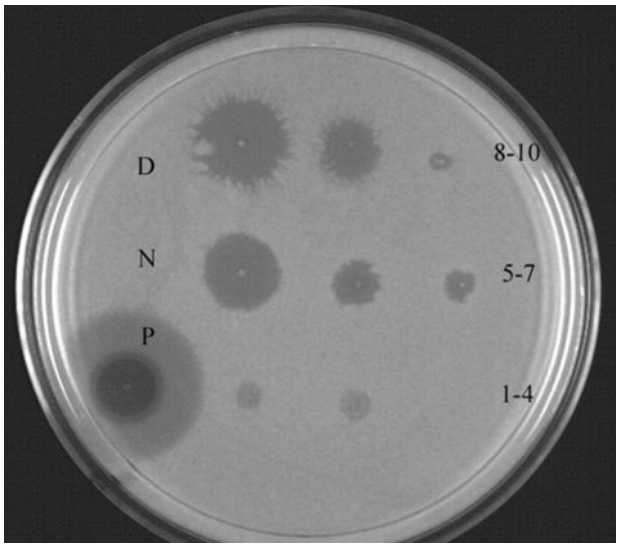

Fig. 3. **Antibacterial activity of *Ginkgo biloba* compounds.** *Salmonella enterica* serovar Typhimurium (10^8 CFU ml^{-1}) was inoculated into 8 ml of molten TSA, and poured over TSA plates. Serial dilutions of compounds 8-10, 5-7, and 1-4 were spotted on the plate. Controls were DMSO (D), Nutrient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

Salmonellosis continues to be a major public health problem worldwide and *Salmonella enterica* serovar Typhimurium is one of the most prevalent serovars among *Salmonella* spp. causing gastroenteritis [20]. 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* serovar Typhimurium phage type DT104, resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines [21]. *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 [22]. 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 *Bifidobacterium bifidum* or *Lactobacillus acidophilus*. Boonkaew and Camper [14] reported that methanolic extracts from leaf and root tissue of

Ginkgo biloba showed no inhibitory activity, but extracts from leaf and root derived callus inhibited the growth of *Klebsiella pneumoniae*, *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*. Finally, 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.

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* serovar Typhimurium and *Listeria monocytogenes*. Moreover, *Ginkgo biloba* compounds 5-7 and 8-10 were also inhibitory towards other important food-borne pathogens, such as *Shigella dysenteriae*, *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 [23-24]. Reports on resistance in *Listeria* spp. have been previously published [25-27]. 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.

REFERENCES

1. Nakanishi K. Terpene trilactones from *Gingko biloba*: from ancient times to 21st century. Bioorgan Med Chem. 2005;13:4987-5000.

2. Leistner E, Drewke C. *Ginkgo biloba* and ginkgotoxin. J Nat Prod. 2010;73:86-92

3. Kleijnen J, Knipschild P. *Ginkgo biloba*. Lancet 1992;340:1136-1139.

4. Cho H-J and Nam K-S. Inhibitory effect of ginkgolide B on platelet aggregation in a cAMP- and cGMP-dependent manner by activated MMP-9. J Biochem Mol Biol. 2007;40:678-83.

5. Soholm B. Clinical improvement of memory and other cognitive functions by *Ginkgo biloba*: review of relevant literature. *Adv Ther.* 1998;**15**:54-65.

6. Drew S and Davies E. Effectiveness of *Ginkgo biloba* in treating tinnitus: double blind, placebo controlled trial. *Brit Med J* 2001;**322:**73.

7. Sakakibara H, Ishida K, Grundmann O, Nakajima J, Seo S, Butterweck V, et al. Antidepressant effect of extracts from *Ginkgo biloba* leaves in behavioral models. Biol Pharm Bull. 2006:29:1767-1770.

8. Gorby HE, Brownawell AM, Falk MC. Do specific dietary constituents and supplements affect mental energy? Review of the evidence. Nutr Rev. 2010;68:697-718.

9. Zhou W, Chai H, Lin PH, Lumsden AB, Yao Q, Chen C. Clinical use and molecular mechanisms of action of extract of *Ginkgo biloba* leaves in cardiovascular diseases. Cardiovas Drug Rev. 2004;22:309-319.

10. Liu TJ, Yeh YC, Ting CT, Lee WL, Wang LC, Lee HW, Wang KY, Lai HC, Lai HC. *Ginkgo biloba* extract 761 reduces doxorubicin-induced apoptotic damage in rat hearts and neonatal cardiomyocytes. Cardiovasc Res. 2008;80:227-235.

11. Atzori C, Bruno A, Chichino G, Bombardelli E, Scaglia M, Ghione M. Activity of bilobalide sesquiterpene from *Ginkgo biloba* on *Pneumocystis carinii*. Antimic Agents Ch. 1993;37:1492-1496.

12. Mazzanti G, Mascellino MT, Battinelli L, Coluccia D, Manganaro M, Saso L. Antimicrobial investigation of semipurified fractions of *Ginkgo biloba* leaves. J Ethnopharmacol. 2000;71:83-88.

13. Lee H-S and Kim M-J. Selective responses of three *Ginkgo biloba* leaf-derived constituents on human intestinal bacteria. J Agr Food Chem. 2002;50:1840-1844.

14. Boonkaew T, Camper ND. Biological activities of Ginkgo extracts. Phytomedicine 2005;12:318-323.

15. Sawano Y, Miyakawa T, Yamazaki H, Tanokura M, Hatano K. Purification, characterization, and molecular gene cloning of an antifungal protein from Ginkgo biloba seeds. Biol Chem. 2007;388:273-80.

16. Sati SC. and Joshi S. Antibacterial activities of *Ginkgo biloba* L. leaf extracts. ScientificWorldJournal. 2011;11:2241-2246.

17. Lee JS, Cho YS, Park EJ, Kim J, Oh WK, Lee HS, Ahn JS. Phospholipase Cγ1 inhibitory principles from the sarcotestas of *Ginkgo biloba*. J Nat Prod. 1998;61:867-871.

18. Carraturo A, Raieta K, Ottaviani D, Russo GL. Inhibition of *Vibrio parahaemolyticus* by a bacteriocin-like inhibitory substance (BLIS) produced from *Vibrio mediterranei* 1. J App Microbiol 2006;101:234-241.

19. EUCAST (European Committee on Antimicrobial Susceptibility Testing). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. Eucast discussion document E.Dis 5.1. 2003. Accessed: 19 December 2012. Available: http://www.escmid.org/fileadmin/src/media/PDFs/2News_Discussions/3Discussion_Documents/E_Def_5_1_03_2003.pdf

20. Pui CF, Wong WC, Chai LC, Lee HY, Noorlis A, Zainazor TC, et al. Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium. Trop Med Health. 2011;39:9-15.

21. World Health Organization Drug-resistant Salmonella. Fact sheet N°139. 2005. Accessed: 19 November 2011. Available: http://www.who.int/mediacentre/factsheets/fs139/en/

22. Ryser ET. Foodborne listeriosis. In *Listeria, Listeriosis and Food Safety* ed. Ryser, E.T. and Marth, E.H. pp. 299-358. Madison: Marcel Dekker Inc.; 1999.

23. Harbarth S and Samore MH. Antimicrobial resistance determinants and future control. Emerg Infect Dis. 2005;11:794-801.

24. Tega L, Raieta K, Ottaviani D, Russo GL, Blanco G, and Carraturo A. Catheter-related bacteremia and multidrug-resistant *Acinetobacter lwoffii*. Emerg Infect Dis. 2007;13:355-356.

25. Abrahim A, Papa A, Soultos N, Ambrosiadis I, Antoniadis A. Antibiotic resistance of *Salmonella* spp. and *Listeria* spp. isolates from traditionally made fresh sausages in Greece. J Food Protect. 1998;61:1378-1380.

26. Walsh D., Duffy G., Sheridan JJ, Blair IS, McDowell DA. Antibiotic resistance among *Listeria*, including *Listeria monocytogenes*, in retail foods. *J Appl Microbiol.* 2001;90:517-522.

27. Conter M, Paludi D, Zanardi E, Ghidini S, Vergara A, Ianieri A. Characterization of antimicrobial resistance of foodborne *Listeria monocytogenes. Int J Food Microbiol. 2009;128:497-500.*