J Food Sci Nutr
Vol 12, p 135~140 (2007)

J Food Science and Nutrition

Antibacterial Activities of Phenolic Components from *Camellia sinensis* L. on Pathogenic Microorganisms

Jung-Sook Shin and Ha-Sook Chung†

Department of Foods and Nutrition, College of Natural Sciences, Duksung Women's University, Seoul 132-714, Korea

Abstract

Antibacterial activities of the major phenolic components from *Camellia sinensis* L. were investigated against several pathogenic microorganisms including Gram-positive strains like *Staphylococcus aureus* ATCC 29213 and *Streptococcus pyogens* 308A; and Gram-negative strains like *Escherichia coli* ATCC 25922, *Escherichia coli* 078, *Pseudomonas aeruginosa* 9027, and *Enterobacter cloacae* 1321E. The MIC values demonstrate that both (-)-epicatechin and (-)-epigallocatechin were more considerably toxic against *Staphylococcus aureus* ATCC 29213 than the other two catechins like (-)-epicatechingallate and (-)-epigallocatechin-3-gallate. (-)-Epicatechingallate and (-)-epigallocatechin-3-gallate were most inhibitory against *Escherichia coli* ATCC 25922. As a result, (-)-epicatechin showed predominant antibacterial activities among tea varieties. The contents of major polyphenolic components such as four catechins, theaflavin, and quercetin were different according to fermentation processes. The total contents of four catechins were ranged from 13.81 to 1.33%, with (-)-epigallocatechin-3-gallate being dominant among tea varieties; theaflavin was found the characteristic pigment in fully-fermented black tea.

Key words: *Camellia sinensis* L., polyphenolic compounds, Gram-negative, Gram-positive, antibacterial activity, fermentation

INTRODUCTION

Bioactive components, as secondary metabolites, can represent extra-nutritional activities with small quantities (1). Polyphenolic compounds in natural plant sources were derived from phenylalanine and tyrosine (2). Most polyphenolic compounds have two or more hydroxyl substituents, including functional derivatives.

Almost all phenolic compounds, as well as polyphenolic compounds, possess several biological and chemical properties including antioxidant activity, cardiovascular disease protection activity, neurodegenerative disorder protection activity, and cancer chemopreventive activity (3).

Such phenolic acids as apigenin, apigenin-7-glucoside, genkwanin, 5-hydroxy-7,4'-dimethoxyflavone, rhamnocitrin, kaempferol, quercetin, quercetin-5,3-dimethylether, luteolin, luteolin-7-glucoside, rhamnazin and scandenone, and anthocyanins showed antimicrobial activities against Gram-positive, Gram-negative bacteria, fungi, and yeast at low concentrations in published data (4-13).

Camellia sinensis L. have been used as a daily beverage and crude medicine in Asia for thousands of years. The major polyphenolic compounds in fresh leaves of *Camellia sinensis* L. are flavan-3-ols that include major catechins, (-)-epicatechin (EC), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG). Polyphenolic compounds of *Camellia sinensis* L. exhibit numerous biological properties including antioxidative effects, inhibition of extracellular mitotic signals through blocking growth receptors signaling, suppression of iNOS through inhibiting the activation of IKK and NFkB, and induction of apoptosis in cancer cells through releasing cytochrome C and activating caspase cascades (14). Processing with non-fermented green tea, differing from fully-fermented black tea, is deactivated by steaming before macerating harvested fresh leaves of *Camellia sinensis* L. The processing is important not only from an economic perspective of bulk commodities but also from a dietary perspective. The total contents of major polyphenolic compounds is various using different manufacturing processes (15).

The aim of this study is to differentiate the contents of major polyphenolic compounds among tea varieties, and also to evaluate the antibacterial activities of major polyphenolic compounds presented in non-fermented, semi-fermented, and fully-fermented tea varieties against several pathogenic microorganisms including Gram-positive strains, *Staphylococcus aureus* ATCC 29213 and *Streptococcus pyogens* 308A and Gram-negative strains,

†Corresponding author. E-mail: hasook@duksung.ac.kr
Phone: +82-2-901-8593, Fax: +82-2-901-8442

Escherichia coli ATCC 25922, *Escherichia coli* 078, *Pseudomonas aeruginosa* 9027, and *Enterobacter cloacae* 1321E.

MATERIALS AND METHODS

Preparation of samples

Fresh leaves of *Camellia sinensis* L. were collected in Hadong, Korea, in 2006. Green tea, prepared through non-fermented tea processing method, was deactivated by hot broiled-bath frying before macerating harvested fresh leaves (Fig. 1). Chung tea (10%-fermented tea) and black tea (85%-fermented tea) were purchased at traditional tea stores in Ankookdong, Seoul, Korea, in 2006.

Strain collections

Collected strains from the Korean Culture Center of Microorganisms (KCCM) were Gram-positive strains like *Staphylococcus aureus* ATCC 29213 and *Streptococcus pyogens* 308A; and Gram-negative strains like *Escherichia coli* ATCC 25922, *Escherichia coli* 078, *Pseudomonas aeruginosa* 9027, and *Enterobacter cloaceae* 1321E.

Instrumental analyses

A Hewelett Packard Model 5985B gas chromatography (GC)/mass (MS) system was used for electron impact mass spectrometry (EI-MS), and negative FAB-MS spectrometry was performed using a JMS-700 spectrometer. The UV/Vis spectrometer was recorded on a Hitachi 3100 UV/Vis spectrophotometer. A Bruker AMX5000 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) with tetramethylsilane (TMS) as an internal standard and DMSO-d_6 as a NMR solvent. Thin-layer chromatographic (TLC) analysis was conducted on silica gel (Kieselgel 60 F_{254} plates; 0.25 mm layer thickness; Merck, Darmstadt, Germany). Silica gel (Merck 60 A, 230～400 mesh ASTM) and Sephadex LH-20 (25～100 μm; Pharmacia Fine Chemicals, Piscataway, NJ, USA) were used for open column and vacuum column for chromatographic separation. All chemicals were purchased from commercial sources and were of the highest purity available.

Isolation and structure elucidation of compounds

Dried, fully ground black tea (1.0 kg) were extracted using 80% (v/v) ethanol (EtOH), three times for five hours in a hot water bath to give ethanolic extracts. The ethanolic extracts were partitioned between ethylacetate (EtOAc) and water. The dried EtOAc-soluble fraction was chromatographed over a silica gel column using a chloroform ($CHCl_3$)-methanol (MeOH) gradient to give twelve fractions. Fraction 3 was further chromatographed on a silica gel open column using $CHCl_3$-MeOH (92:8 to 89:11, v/v) to give compounds 1, 3 and 6. Fractions 6 and 7 were combined and further chromatographed on a silica gel vacuum column using a $CHCl_3$-MeOH-H_2O (95:5:0.5, v/v), and subfractions 26-41 were rechromatographed on a Sephadex LH-20 column by elution with MeOH in order to give pale yellow powder. This powder was further chromatographed on a silica gel vacuum column and Sephadex LH-20 column by elution with aqueous EtOAc-MeOH (93:7) and MeOH, respectively, to give compounds 2, 4 and 5. Complete identification of isolated compounds made use of varieties of physical and chemical techniques by UV/Vis spectrophotometry, EI-MS spectrometry, negative FAB-MS spectrometry, ^{1}H-NMR and ^{13}C-NMR spectroscopy.

Quantification of major phenolic compounds among tea varieties

Such dried green tea as chung tea and black tea (100 g) were extracted with 80% EtOH and concentrated. Each concentrations of major phenolic compounds among tea varieties were identified in all ethanolic extracts by HPLC using YMC C18 column (10×250 mm, Hitachi D-7000, Japan) and a mobile phase consisting of water-acetonitrile-methanol-water (80:17.5:1.5:1.0, v/v/v/v).

Antibacterial activity on pathogenic microorganisms

The antibacterial activities were evaluated by determining the minimum inhibitory concentration (MIC) according to the M7-A5 guidelines, established by the

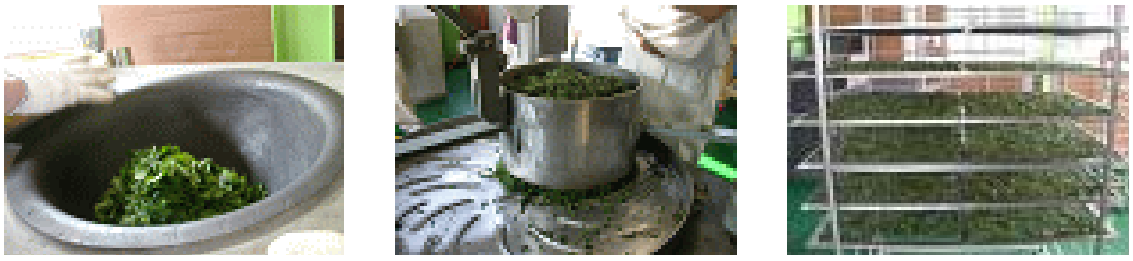


Fig. 1. Preparation of green tea from fresh leaves of *Camellia sinensis* L.

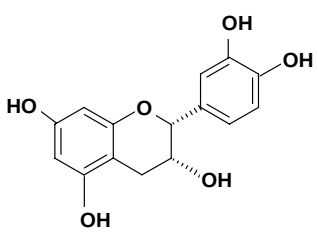
National Committee for Clinical Laboratory Standards (16). The MIC values of the bacterial strains against Gram-positive strains like *Staphylococcus aureus* ATCC 29213 and *Streptococcus pyogens* 308A; and Gram-negative strains like *Escherichia coli* ATCC 25922, *Escherichia coli* 078, *Pseudomonas aeruginosa* 9027, and *Enterobacter cloaceae* 1321E were determined on 96 well culture plates by a micro dilution method (17). Phenolic compounds 1～6 were serially diluted with EtOH, and Tween 80 was added to each solution. Each diluted solution was carried out from the concentration of 0.5% w/v (5% of Tween 80, used to dissolve the compounds and thus to ensure its contact with microorganisms). All preparations were sterilized with a 0.22 μm filter. The wells were inoculated with a microorganism suspension at the density of 10^5 cells/mL. The plates were incubated at 37°C for 24 hr. Followed by the incubation, the plates were observed to determine the MIC Proper blanks were prepared simultaneously, and compounds 1～6 were tested in triplicate.

RESULTS AND DISCUSSION

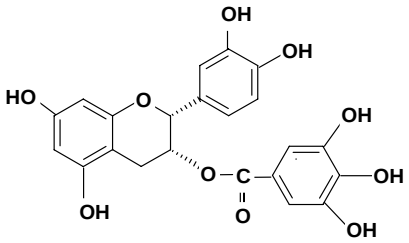
Isolation and structure elucidation of compounds 1～6

Green tea, as a non-fermented tea variety, was deactivated by hot broiled-bath frying before macerating harvested fresh leaves (Fig. 1). Commercial chung tea (10%-fermented tea) and black tea (85%-fermented tea) were purchased from traditional tea stores.

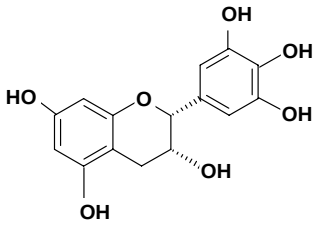
The dried, fully ground black tea (1.0 kg) was extracted using 80% (v/v) EtOH, three times for five hours in a hot water bath to give ethanolic extracts. The ethanolic extracts were partitioned between EtOAc and water. The concentrated EtOAc-soluble fraction was chromatographed over a silica gel column using a $CHCl_3$-MeOH to give twelve fractions. Fraction 3 was further chromatographed on a silica gel open column using $CHCl_3$-MeOH (92:8 to 89:11, v/v) to give compounds 1, 3 and 6. Fractions 6 and 7 were combined and further chromatographed on a silica gel vacuum column to give compounds 2, 4 and 5. Compounds were monitored by TLC patterns revealed by a UV lamp and 10% $FeCl_3$-MeOH spray. Complete identification of the isolated compounds made use of various physical and chemical methods, including EI-MS, negative FAB-MS, UV/Vis, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. All compounds gave characteristic phenolic color reactions (purplish brown with $FeCl_3$, yellow with NaOH, yellowish orange with Mg-HCl and pink with Zn-HCl). The structures of the compounds were identified by comparing spectral data with published data (3,18). The isolated compounds were determined as (-)-epicatechin (EC, 1), (-)-epicatechingallate (ECG, 2), (-)-epigallocatechin (EGC, 3), (-)-epigallocatechin-3-gallate (EGCG, 4), theaflavin (5) and quercetin (6) (Fig. 2).



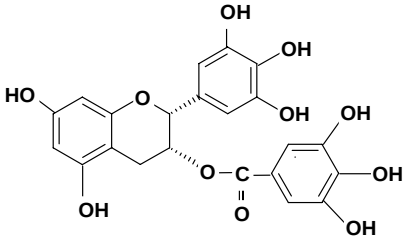
1: (-)-Epicatechin



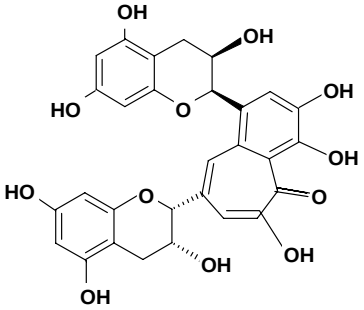
2: (-)-Epicatechingallate



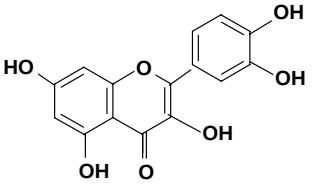
3: (-)-Epigallocatechin



4: (-)-Epigallocatechin-3-gallate



5: Theaflavin



6: Quercetin

Fig. 2. Chemical structure of major phenolic compounds from *Camellia sinensis* L.

Quantification of major phenolic compounds among tea varieties

HPLC data of the individual compounds in ethanolic extracts of green tea, chung tea, and black tea revealed remarkably high major catechins. The contents of major catechins ranged from 13.81 to 1.33%, with compound 4 being dominant among tea varieties. Remarkably, non-fermented tea, green tea contained a high concentration of total catechins 1～4, while fermented teas had shown the reduction of total catechins 1～4 and increase of compound 5 contents (Table 1). It can be explained that the fermentation of tea leaves induces enzymatic oxidation of flavan-3-ols and leads to the formation of major pigments, theaflavins, in black tea. Compound 5 can be synthesized by fermentation procedure by coupled oxidation mechanism, and encompassed molecules containing a benzotropolone nucleus formed by an oxidative reaction between the *vic*-trihydroxybenzene and *ortho*-dihydroxybenzene of catechins. It is formed by oxidative coupling of the B-ring (3',4',5'-trihydroxyl) of compound 3 or compound 4 and B-ring (3',4'-dihydroxyl) of one compound 1 or compound 2. It has been ionized by protonation and formation of adduct ions, classically described for flavan-3-ols and proanthocyanidin derivatives by MAL.DI-TOF analysis (18).

Table 1. Concentrations (%) of major phenolic compounds from non-fermented, semi-fermented and fully-fermented tea varieties

Compounds	Tea varieties		
	Green tea[1)]	Chung tea[2)]	Black tea[3)]
(-)-Epicatechin	0.71	0.38	0.10
(-)-Epicatechingallate	3.49	2.07	0.10
(-)-Epigallocatechin	2.10	1.92	0.46
(-)-Epigallocatechin-3-gallate	7.51	4.92	0.67
Theaflavin	-	-	2.10
Quercetin	0.40	0.22	0.09

[1)]Non-fermented tea. [2)]Semi-fermented tea. [3)]Fully-fermented tea.

Compound 5, the most important phenolic pigments in black tea exhibited good inhibition rates of approximately 85～90% for antioxidant and scavenging activities of free radicals and protected COS-7 cells against apoptosis or damage caused by stress, such as cadmium and copper-oxidative injury, free radicals (19). Recently, the coupled oxidation mechanism for synthesis are presented (Fig. 3). Upon enzymatic synthesis of compound 5 from compound 1 and 2, trapping of the *O*-quinone intermediates with glutachione demonstrated that enzymes rapidly oxidized compound 1 and 3 to EC-quinone (1a) and EGC-quinone (2a), respectively (20).

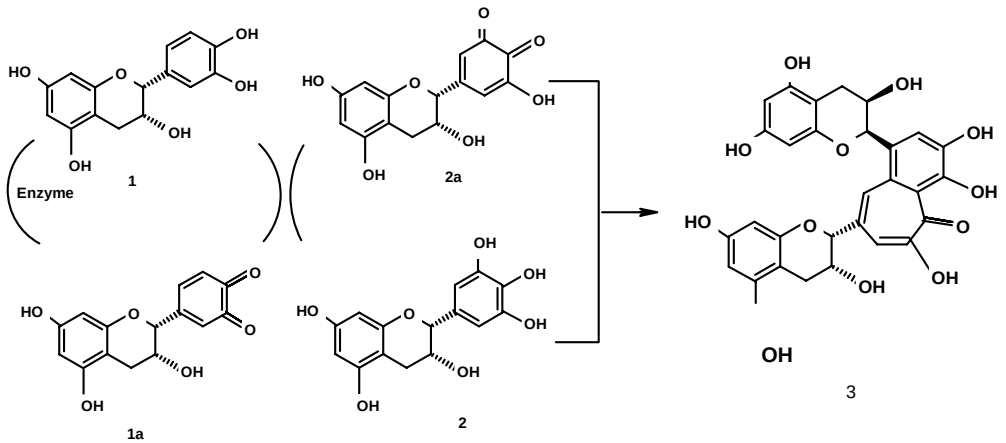


Fig. 3. Coupled oxidation mechanism for theaflavin synthesis.

Table 2. Antibacterial activity of major phenolic compounds from *Camellia sinensis* L. on pathogenic microorganisms[1)]

Strains	MIC (μg/mL)					
	1	2	3	4	5	6
Staphylococcus aureus ATCC 29213	12.5	50	12.5	100	100	200
Streptococcus pyogens 308A	50	25	50	100	200	100
Escherichia coli ATCC 25922	100	12.5	50	12.5	100	100
Escherichia coli 078	50	50	50	25	200	100
Pseudomonas aeruginosa 9027	25	50	50	50	100	200
Enterobacter cloacae 1321E	50	12.5	25	100	100	200

[1)]Results are expressed as MIC (μg/mL).

Antibacterial activity of compounds 1～6

In the present study, the antibacterial activities of major polyphenolic compounds 1～6 were evaluated. As presented in Table 2, Each of the compounds inhibits the growth of the microbial strains including *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogens* 308A, *Escherichia coli* ATCC 25922, *Escherichia coli* 078, *Pseudomonas aeruginosa* 9027, and *Enterobacter cloacae* 1321E. The MIC values demonstrate that compounds 1 and 3 were more considerably toxic against *Staphylococcus aureus* ATCC 29213 than compounds 2 and 4. Compounds 2 and 4 were inhibitory against *Escherichia coli* ATCC 25922. Compound 2 was more active against Gram-positive bacteria than Gram-negative bacteria. This is likely due to the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. The modes of action of bacterial agents depend on the types of microorganisms and are mainly related to both cell wall structures and outer membrane arrangements of microorganisms. Gram-negative bacteria possess an outer membrane and a unique periplasmic space found in no Gram-positive bacteria (21). The resistance of Gram-negative bacteria toward antibacterial substances is related to the hydrophilic surface of their outer membranes which are rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules. Such resistance, also, is associated with the enzymes in the periplasmic space, which are capable of breaking down into the molecules introduced from outside (22). Otherwise, Gram-positive bacteria would not have such outer membranes and cell wall structures. Antibacterial substances can easily destroy the bacterial cell walls and cytoplasmic membranes, resulting in a leakage of the cytoplasm and its coagulation (23).

It had already demonstrated that green tea showed increased antimicrobial activities against bacteria and fungi when used in combination with butylated hydroxyanisole. The increased antimicrobial activity of green tea was related to an impairment of the barrier function in microorganisms and a depletion of thiol groups. The increased role of green tea as an oral antimicrobial product was discussed (24).

Plants with antibacterial activities have become more interesting, some of which are part of the arsenal of modern antibacterial nutraceuticals and drugs. Many people are increasingly aware of problems associated with the over-prescription and misuse of traditional antibiotics.

In this study, the antibacterial activities of phenolic compounds from non-fermented tea and fermented tea was investigated for the supplementation in anti-biotic nutraceutical preparation and daily uses. Further research is required to provide more *in vivo* data to identify the antibacterial mechanisms of major compounds of tea varieties.

ACKNOWLEDGEMENT

This research was supported by Institute of Natural Sciences, Duksung Women's University, Seoul, Korea (2006).

REFERENCES

1. Han HK, Choi SS, Kim YR, Kim HJ, Kang GM, Dong MS, Na CS, Chung HS. 2006. Diarylheptanoid and flavonoid with antioxidant activity from *Alnus japonica* Steud on DPPH free radical scavenging assay. *J Food Sci Nutr* 11: 171-175.
2. Shahidi F, Naczk M. 2004. *Phenolics in Food and Nutraceuticals*. CRC Press, Boca Raton, FL, USA.
3. Chung HS, Chang LC, Lee SK, Shamon LA, van Breemen RB, Mehta RG, Farnsworth NR, Pezzuto JM, Kinghorn AD. 1999. Flavonoid constituents of *Chorizanthe diffusa* with potential cancer chemopreventive activity. *J Agric Food Chem* 47: 36-41.
4. Martini ND, Katerere DR, Eloff JN. 2004. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *J Ethnopharmacol* 93: 207-212.
5. Sousa A, Ferreira IC, Calhelha R, Andrade PB, Valentao P, Seabra R, Estevinho L, Bento A, Pereira JA. 2006. Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. *Bioorg Med Chem* 14: 8533-8538.
6. Suzgec S, Mericli AH, Houghton PJ, Cubukcu B. 2005. Flavonoids of Helichrysum and their antioxidant and antibacterial activity. *Fitoterapia* 76: 269-272.
7. Ozcelik B, Orhan I, Toker G. 2006. Antiviral and antimicrobial assessment of some selected flavonoids. *Z Naturforsch* 61: 632-638.
8. Leitao DP, Polizello AC, Ito IY, Spadaro AC. 2005. Antibacterial screening of anthocyanic and proanthocyanic fractions from cranberry juice. *J Med Food* 8: 36-40.
9. Zhou L, Li D, Wang J, Liu Y, Wu J. 2007. Antibacterial phenolic compounds from the spines of *Gleditsia sinensis* Lam. *Nat Orod Res* 21: 283-291.
10. Kuete V, Simo IK, Ngameni B, Bigoga JD, Watchueng J, Kapguep RN, Etoa FX, Tchaleu BN, Beng VP. 2007. Antimicrobial activity of the methanolic extract, fractions and four flavonoids from the twigs of *Dorstenia angusticornis* Engi. (Moraceae). *J Ethnopharmacol* 112: 271-277.
11. Phadungkit M, Luanratana O. 2006. Anti-Salmonella activity of constituents of *Aradisia elliptica* Thunb. *Nat Prod Res* 20: 693-696.
12. Takahashi T, Kokubo R, Sakaino M. 2004. Antimicrobial activities of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculate*. *Lett Appl Microbiol* 39: 60-64.
13. Cushnie TP, Lamb AJ. 2005. Antimicrobial activity of flavonoids. *Int J Antimicrob Agents* 26: 343-356.
14. Yang CS, Prabhu A, Landau J. 2001. Prevention of carcinogenesis by tea polyphenols. *Drug Met Rev* 33: 237-253.

15. Robertson A. 1992. *Tea: Cultivation to Consumption*. Wilson KC, Clifford MN, eds. Chapman and Hall, London. p 555-601.
16. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard M-7-A5; National Committee for Clinical Laboratory Standard, 2000. Villanova, PA, USA.
17. Farag RS, Daw ZY, Heweai FM, EL-Bbaroty GSA. 1989. Antimicrobial activity of some Egyptian spice essential oils. *J Food Prot* 52: 665-667.
18. Menet MC, Sang S, Yang CS, Ho CT, Rosen RT. 2004. Analysis of theaflavins and thearubigins from black tea extract by MALDI-TOF Mass Spectrometry. *J Agric Food Chem* 52: 2455-2461.
19. Shon MY, Park SK, Nam SH. 2007. Antioxidant activity of theaflavin and thearubigin separated from Korean microbially fermented tea. *J Food Sci Nutr* 12: 7-10.
20. Tanaka T, Mine C, Watarumi S, Matsuo Y, Kouno I. 2005. Production of theaflavin and theasinensins during tea fermentation. In *Phenolic compounds in foods and natural health products*. Shahidi F, Ho C-T, eds. ACS symposium series 909, American Chemical Society, Washington DC, USA. p 190.
21. Duffy CF. Power RF. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts. *Int J Antimicrob Agents* 17: 527-529.
22. Gao Y, van Belkum MJ, Stiles ME. 1999. The outer membrane of gram-negative bacteria inhibits antibacterial activity of brochocin-C. *Appl Environ Microbiol* 65: 4329-4333.
23. Kalemba D, Kunicka A. 2003. Antibacterial and antifungal properties of essential oils. *Curr Med Chem* 10: 813-829.
24. Simonetti G, Simonetti N, Villa A. 2004. Increased microbicidal activity of green tea (*Camellia sinensis*) in combination with butylated hydroxyanisole. *J Chemother* 16: 122-127.

(Received August 23, 2007; Accepted September 7, 2007)