



Original Article

Determination of reactive oxygen generated from natural medicines and their antibacterial activity[☆]Noriko Tajima^a, Makiko Takasaki^a, Haruka Fukamachi^b, Takeshi Igarashi^b, Yoshiji Nakajima^c, Hidetoshi Arakawa^{a,*}^a Analytical Chemistry, School of Pharmacy, Showa Univ., 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan^b Oral Microbiology and Immunology, School of Dentistry, Showa Univ., 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan^c Ohta's Isan Co., Ltd., 957 Shishikocho, Ushiku, Ibaraki 300-1231, Japan

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ABSTRACT

Extracts of 16 natural medicine powders (*Galla chinensis*, *Malloti cortex*, *Cassiae semen*, *Sophorae radix*, *Myricae cortex*, *Crataegi fructus*, *Gambir*, *Mume fructus*, *Geranii herba*, *Phellodendri cortex*, *Coptidis rhizoma*, *Swertiae herba*, and *Cinnamomi cortex*) were assayed for reactive oxygen concentrations using the peroxyoxalate chemiluminescent detection system. High luminescence intensity was observed in *Galla chinensis*, *Geranii herba*, *Malloti cortex*, *Myricae cortex*, and *Cinnamomi cortex*. Additional experiments identified the reactive oxygen species as hydrogen peroxide.

Galla chinensis generated 2.4×10^{-4} mol/L hydrogen peroxide from a 1 mg/mL solution. In bacterial growth tests, *Galla chinensis* extract had antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacteroides thetaiotaomicron*, *Campylobacter sputorum biovar sputorum*, *Streptococcus salivarius thermophilus*, *Lactobacillus casei*, and *Bifidobacterium longum infantis*. This antibacterial activity was decreased by the addition of catalase. It revealed that hydrogen peroxide which *Galla chinensis* produced participated in antibacterial activity.

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1. Introduction

Natural medicines that act on the stomach are used for the treatment of illnesses of the stomach and intestines, such as diarrhea. The mechanism of pharmacological action differs for each medicine. For example, berberine, a component of *Malloti cortex*, has an inhibitory effect on ulcers [1]; *Geranii herba* induces constipation by inhibiting intestinal peristalsis [2]; and *Coptidis rhizoma* contains berberine and coptisine, components with demonstrated bactericidal activity [3].

It is already known that the reactive oxygen contributes to the bactericidal action. For example, the white blood cells and macrophages in the blood use the reactive oxygen in order to kill the bacteria that have invaded the body. In addition, the aqueous solution of hydrogen peroxide is used as a disinfectant. We revealed previously that tea and persimmon have the bactericidal activity because of the hydrogen peroxide generated from their components [4–6]. In the case of tea, the production of hydrogen peroxide in its extracts probably involves a reaction between

dissolved oxygen and catechin. Various physiological effects of catechins are well known, and the antibacterial and antitoxin effects are important physiological functions. For instance, catechins can contribute to the improvement of the intestinal flora [7,8], sterilization of *Vibrio cholerae*, and the inhibition of the effects of pertussis toxin and verotoxin of pathogenic *E. coli* [9,10]. Gastrointestinal (GI) disorders are often caused by bacteria, the growth and toxin production of food-poisoning bacteria, and imbalances in intestinal flora.

Many natural medicines acting on the GI system contain polyphenols and polymers thereof, such as catechin and tannin [11,12]. The bactericidal effects of tannins against intestinal bacteria may contribute to the relief of GI disorders.

In this study, we characterized 16 species of natural medicines that have been reported to act on the GI tract. First, we identified and quantified reactive oxygen species (ROS) generated from these medicines. Second, we examined the antibacterial activity of the natural medicines for eight bacterial strains, including four types that cause food poisoning or opportunistic infections, and the other four types (such as lactic acid bacteria) involved in GI homeostasis. Third, we investigated the relationship between ROS generated from natural medicines and associated antibacterial action.

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2. Materials

2.1. Reagents

Reagents were obtained from the following suppliers: hydrogen peroxide (30%) and iron (II) sulfate heptahydrate from Wako Pure Chemical Industries Ltd. (Osaka, Japan); bis(2,4,6-trichlorophenyl) oxalate (TCPO) from Tokyo Chemical Industries Inc. (Tokyo, Japan); 8-anilinoanthralene sulphonate ammonium salt (ANS) from Merck (Darmstadt, Germany); diethylenetriamine penta acetic acid (DTPA) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) from Dojindo Laboratories (Kumamoto, Japan); catalase from Roche Diagnostics K.K. (Tokyo, Japan); bovine serum albumin (BSA) from Sigma-Aldrich (St Louis, U.S.A.). All other chemicals were obtained as reagent-grade.

The natural medicine powders were donated by Ota's Isan Co., Ltd. (Ibaraki, Japan), and included the following: *Galla chinensis*, *Malloti cortex*, *Cassiae semen*, *Sophorae radix*, *Myricae cortex*, *Crataegi fructus*, *Gambir*, *Mume fructus*, *Geranii herba*, *Phellodendri cortex*, *Coptidis rhizoma*, *Swertiae herba*, and *Cinnamomi cortex* (obtained from four sources as Dongxing, Indonesia, Guangnan and Vietnam).

2.2. Reagent preparation

Natural medicine powders were suspended at 1 mg/mL in 0.1 mol/L phosphate buffer (pH 7.0) and incubated for 10 min at 60 °C. The suspensions were filtered (pore size 0.45 µm) before use.

2.3. Peroxyoxalate chemiluminescence detection for hydrogen peroxide

Prior to the assay, an ANS solution was prepared by dissolving ANS (20 mg) and BSA (0.1 g) in 100 mL of 0.2 mol/L barbital buffer (pH 9.0). This ANS solution was stored at 4 °C for pending use. The filtered suspension of natural medicine (100 µL) and 0.1 mol/L carbonate buffer (pH 10.0, 900 µL) were mixed and incubated for 30 min at 37 °C. As a blank, the natural medicine suspension was replaced with 100 µL of 0.1 mol/L phosphate buffer (pH 7.0). Following incubation, the sample solution (100 µL) was mixed with 100 µL ANS solution and 200 µL of 5 mmol/L TCPO in ethyl acetate. After a 15 s wait, luminescent intensity was measured for a 10 s interval using a Luminescence Reader BLR-301 (Hitachi Aloka Medical Ltd., Tokyo, Japan). The measurements were performed in triplicates and the results were shown as the average.

2.4. Identification of reactive oxygen

Extract solutions of natural medicines (100 µL), 0.1 mol/L carbonate buffer (pH 10.0, 900 µL), and catalase (1300 U/mL, 10 µL) were mixed and incubated for 30 min at 37 °C. The intensity of luminescence then was measured as described above.

2.5. Measurement of ESR spectrum

Electron spin resonance (ESR) reactions were generated by serial combination of 160 µL of 50 mmol/L phosphate buffer (pH 7.4), 30 µL of 0.5 mmol/L ferrous sulfate, 30 µL of 1 mmol/L DTPA, 50 µL of ultra-pure water, 5 µL of DMPO, and 30 µL of filtered natural medicine suspension. For generation of the standard curve, the natural medicine suspension was replaced with an equivalent volume of hydrogen peroxide standard solution. The reaction solution was placed within the ESR cell cavities, and the spectrum was measured using an ESR spectrometer FR30EX (JEOL RESONANCE, Tokyo, Japan).

2.6. Bacterial strains and culture conditions

Bacterial strains were as follows: *Escherichia coli* ATCC10798, *Staphylococcus aureus* ATCC6538, *Bacteroides thetaiotaomicron* ATCC29148, *Campylobacter sputorum* biovar *sputorum* CDC43563, *Enterococcus faecalis* JCM5803, *Streptococcus salivarius thermophilus* JCM20026, *Lactobacillus casei* JCM1134, and *Bifidobacterium longum infantis* JCM1222.

The cell culture media were as follows: Luria-Bertani (LB) broth (20 g/L) for *E. coli*; Bacto Todd-Hewitt broth (37 g/L) for *E. faecalis*, *L. casei*, and *S. thermophilus*; Bacto Brain Heart Infusion broth (BHIB, 37 g/L) for *S. aureus*; BHIB supplemented with NaCl (3 g/L) for *C. sputorum*; BHIB supplemented with hemin (5 µg/mL) and menadione (5 µg/mL) for *B. thetaiotaomicron*; and Gifu Anaerobic Medium (GAM) broth (59 g/L) for *B. longum*. *E. coli* and *S. aureus* were incubated under aerobic conditions (37 °C, 18 h). Other bacteria were incubated under anaerobic conditions (48–72 h) using Anaero Pack-Kenki (Mitsubishi Gas). Where needed, solid medium (agar plates) was prepared by addition of agar (15 g/L) to the respective medium.

2.7. Effect of *Galla chinensis* and hydrogen peroxide on antibacterial activity

2.7.1. Preparation of test samples

Galla chinensis was extracted with phosphate buffer (pH 7.0) as described above. The filtered suspension of *Galla chinensis* (100 µL) was incubated with 0.1 mol/L carbonate buffer (pH 8.0, 900 mL) for 30 min at 37 °C. For the catalase experiment, catalase (100 units, 550 µL) was combined with 1 mL of the sample solution; for the control reactions, the catalase was replaced with an equivalent volume of ultra-pure water.

2.7.2. Preparation of bacterial suspension

Cell cultures were harvested by centrifugation (3000 g, 15 min). The final concentration of cells was adjusted at 1.0×10^5 colony-forming units (CFU)/mL by using phosphate buffer (pH 7.0).

2.7.3. Mixing of the sample and the bacteria

Sample solution (155 µL) and bacterial suspension (845 µL) were mixed and incubated for 18 h at 37 °C. For the catalase effect test, catalase pre-treated sample solution was used. For the controls, the sample solution was replaced with an equivalent volume of buffer solution (negative control) or catalase pre-treatment buffer solution (control for catalase effect test). Following incubation, the sample-bacteria mixtures were subjected to 10-fold serial dilutions and then spread or spotted to agar medium to determine growth, as described below.

2.7.4. Determination of antibacterial effect

The number of viable cells of *E. coli* and *S. aureus* was counted as follows. Samples were subjected to 10-fold serial dilution to 10^{-9} . Aliquots (100 µL) were spread to agar plates and cultured under appropriate conditions. Cultured by three plates per each dilution, the number of viable cells was calculated from the dilution factor and the average number of colonies.

For other bacterial strains, the growth of colonies was observed with the agar spot method. Samples were subjected to 10-fold serial dilution to 10^{-4} . Aliquots (5 µL) were spotted to agar plates and then cultured under appropriate conditions.

Colony density on the plates was scored as follows: + + +, dense growth; + +, density reduced compared to control, but too numerous to count; +, the number of colonies can be counted; –, no colonies.

3. Results and discussion

3.1. Identification and quantification of hydrogen peroxide

Chemiluminescent intensity of each natural medicine extract was measured. The results are shown as signal-to-noise (S/N) ratio of chemiluminescence (Fig. 1). In the 16 species of natural medicine powders that were tested, *Galla chinensis*, *Gambir*, *Myrica cortex*, *Geranii herba* and *Cinnamomi cortex* indicated particularly high luminescence intensity ($S/N > 10$). For all of these cases, the luminescence disappeared upon addition of catalase, indicating that the ROS causing the luminescence was hydrogen peroxide.

For the natural medicines that yielded strong luminescence, hydrogen peroxide concentrations were determined for 1 mg/mL suspensions and the results are shown in Table 1. Peroxide concentrations were calculated based on a calibration curve of a standard solution of hydrogen peroxide. For the 1 mg/mL suspensions, *Galla chinensis* and *Gambir* yielded peroxide $\geq 1 \times 10^{-4}$ mol/L; *Geranii herba*, *Myrica cortex* and the 4 *Cinnamomi cortex* samples yielded peroxide at 1×10^{-4} – 10^{-5} mol/L.

Galla chinensis, which yielded the strongest luminescence in our assay, is a raw material of tannic acid and contains large amounts of tannins and gallic acid [11]. These components are presumed to contribute to the production of hydrogen peroxide.

3.2. Identification of ROS using ESR

ESR measurements were performed by Fenton reaction and DMPO spin trap method.

When detecting hydrogen peroxide by the DMPO spin trap method, we typically observed an ESR spectrum of hydroxyl radical-DMPO adducts (Fig. 2) consisting of 4 lines with an intensity ratio of 1:2:2:1.

As shown in Fig. 2, the ESR spectrum of *Galla chinensis* extract matched the 4-line spectrum diagnostic of hydrogen peroxide. The similar spectra were observed in extracts from *Gambir*, *Myrica cortex*, *Geranii herba*, *Malloti cortex* and *Cinnamomi cortex*. The elimination of the spectra following the addition of catalase confirmed the proposed identity of this component.

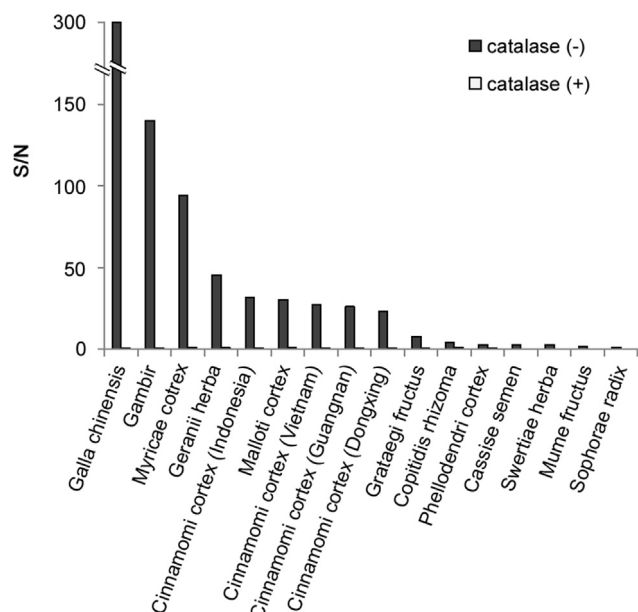


Fig. 1. Relative chemiluminescent intensity of natural medicine extracts.

Table 1

Hydrogen peroxide concentration of natural medicine extracts.

Natural medicine species	H ₂ O ₂ (mol/L)
<i>Galla chinensis</i>	2.4×10^{-4}
<i>Gambir</i>	1.4×10^{-4}
<i>Myrica cortex</i>	7.3×10^{-5}
<i>Geranii herba</i>	3.4×10^{-5}
<i>Cinnamomi cortex</i> (Indonesia)	2.3×10^{-5}
<i>Malloti cortex</i>	2.2×10^{-5}
<i>Cinnamomi cortex</i> (Vietnam)	2.0×10^{-5}
<i>Cinnamomi cortex</i> (Guangnan)	1.8×10^{-5}
<i>Cinnamomi cortex</i> (Dongxing)	1.6×10^{-5}

3.3. The amount of hydrogen peroxide generated from *Galla chinensis*

As described above, among the 16 kinds of natural medicines screened here, *Galla chinensis* generated the highest level of hydrogen peroxide. Therefore, the relationship between the amount of *Galla chinensis* and the generated hydrogen peroxide concentration was examined by chemiluminescent detection. Specifically, *Galla chinensis* was extracted with 0.1 mol/L phosphate buffer solution (pH 7.0), and incubated in 0.1 mol/L carbonate buffer (pH 8.0), similar to the weakly basic intestinal environment encountered by intestinal bacteria. After incubation in carbonate buffer, hydrogen peroxide was quantified by the chemiluminescent method. The results are shown in Fig. 3. At concentrations of *Galla chinensis* from 1 to 10 mg/mL, hydrogen peroxide was generated to be concentration-dependent. Linearity was lost at higher concentrations of *Galla chinensis* (> 10 mg/mL). Thus, the generation of hydrogen peroxide does not depend solely on the concentration of *Galla chinensis*. Possible reasons for this effect include saturation of the *Galla chinensis* or saturation of dissolved oxygen as the dissolved oxygen reacts with the hydroxyl groups of tannins (as provided by *Galla chinensis*) to generate hydrogen peroxide [4,5].

3.4. Hydrogen peroxide sensitivity of bacteria

The hydrogen peroxide sensitivities of bacteria were measured. The results are shown in Table 2. The growth of *E. coli* (a species associated with diarrhea), *S. aureus* and *C. sputorum* (bacteria causing food poisoning), and *B. thetaiotaomicron* (a species that causes opportunistic infections) was inhibited by exposure to hydrogen peroxide at concentrations over 6.0×10^{-4} mol/L. *L. casei*, *S. thermophilus*, and *B. longum* (bacteria contained in yogurt and probiotic products) growth were inhibited by exposure to hydrogen peroxide at concentrations above 1×10^{-2} mol/L. *E. faecalis* growth was not inhibited completely by hydrogen peroxide at concentration ranging from 1.0×10^{-5} – 1.0×10^{-2} mol/L.

3.5. Inhibitory effects of *Galla chinensis* extract on bacterial growth

The *Galla chinensis* extract was incubated with bacteria to examine its antibacterial activity. Before incubation, the hydrogen peroxide concentration of extract was confirmed to be 6.0×10^{-4} mol/L by measurement. The results are shown in Table 3.

As seen with the hydrogen peroxide standard solution, the *Galla chinensis* extract inhibited the growth of *E. coli*, *C. sputorum*, *B. thetaiotaomicron* and *S. aureus*. Notably, growth inhibition was not observed upon exposure to *Galla chinensis* extracts that had been pretreated with catalase, suggesting that the antibacterial activity observed in *E. coli*, *S. aureus*, and *B. thetaiotaomicron* was

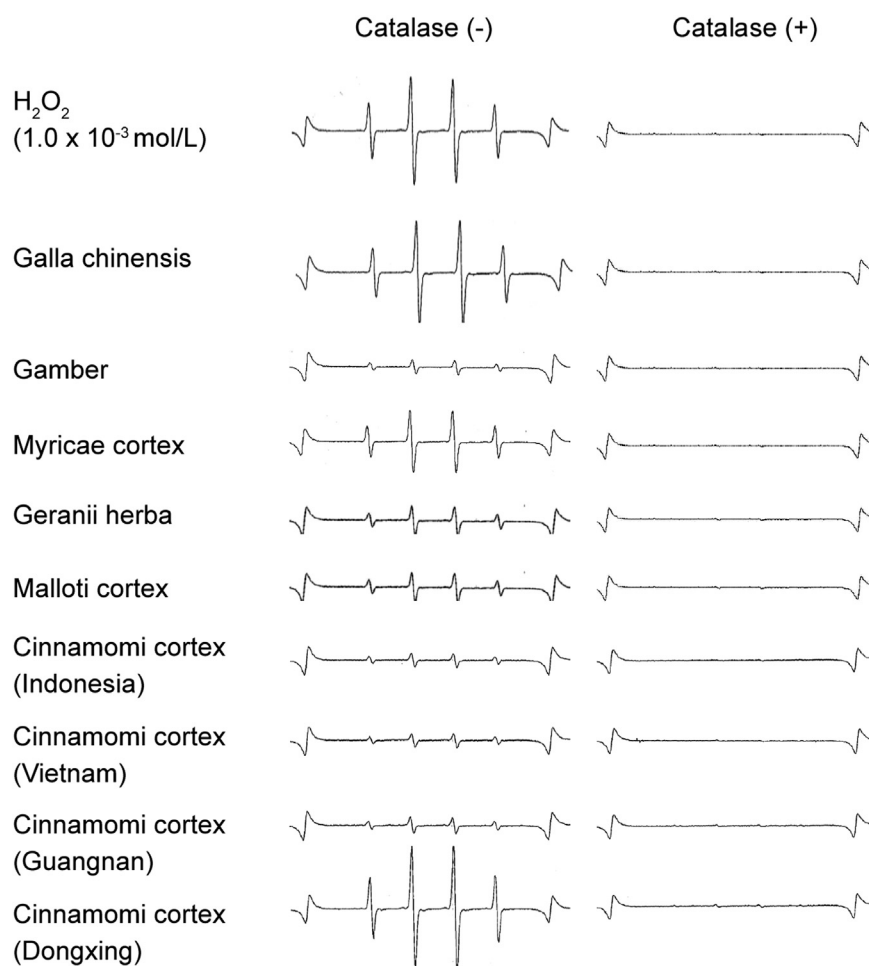


Fig. 2. ESR spectra of H_2O_2 and natural medicines.

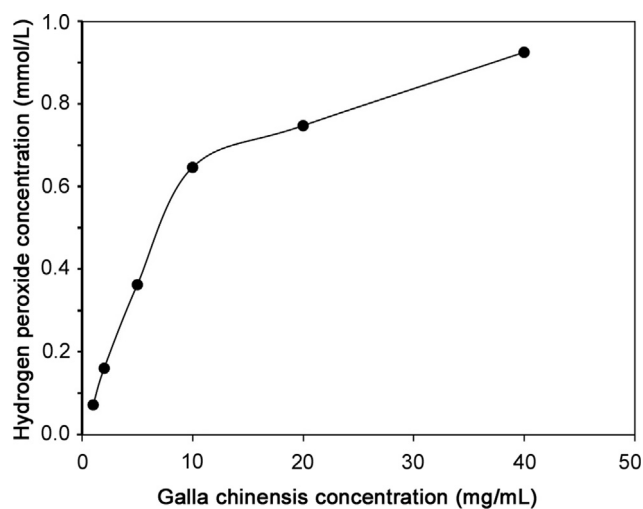


Fig. 3. Concentration of H_2O_2 generated from *Galla chinensis*.

mediated by hydrogen peroxide. In contrast, some growth inhibition of *C. sputorum* was still seen despite catalase pretreatment of the *Galla chinensis* extract. This observation suggests that *C. sputorum* growth inhibition is mediated by some additional (non-peroxide) component(s) of the extract.

Inhibition of *E. faecalis* growth was not seen in the *Galla chinensis* extract, with or without catalase. This result correlated with our results that *E. faecalis* has low sensitivity to hydrogen peroxide.

Galla chinensis extract inhibited the growth of *L. casei*, *S. thermophilus*, and *B. longum*. When *Galla chinensis* extract was pre-treated with catalase, these bacterial growth was restored, although it was less than that of the control. Therefore, growth inhibitory effects against *L. casei*, *S. thermophilus*, and *B. longum* by *Galla chinensis* extract were presumed to involve hydrogen peroxide generated from *Galla chinensis*, since the antibacterial activity was lost upon pretreatment with catalase.

The hydrogen peroxide sensitivity test showed that the minimum growth inhibition concentration of *S. thermophilus* was 5.0×10^{-3} mol/L, and that of *L. casei* and *B. longum* was 1.0×10^{-2} mol/L. In this experiment, *Galla chinensis* extract included the hydrogen peroxide concentration at 6.0×10^{-4} mol/L, despite insufficient concentration required for bactericidal effect, *L. casei*, *S. thermophilus*, and *B. longum* growth was inhibited at this concentration. It is thought that the discrepancy indicates that *Galla chinensis* extract may contain antibacterial component(s) other than hydrogen peroxide, or may contain a component that enhances the action of hydrogen peroxide against *L. casei*, *S. thermophilus*, and *B. longum*. This additional *Galla chinensis* component was not inactivated by catalase, given that catalase-treated *Galla chinensis* extract reduced the number of bacteria below that seen with the buffer control.

In our previous studies on the bactericidal activity of catechins, we observed that protein aggregation by tannins affected the bactericidal activity of reactive oxygen generated by catechin [5]. In addition, other researchers have reported that bacterial cell membranes were destroyed upon exposure to a fungal

Table 2
Effect of H₂O₂ on bacterial strains.

H ₂ O ₂ (mol/L)	<i>E. faecalis</i>	<i>S. thermophilus</i>	<i>L. casei</i>	<i>B. longum</i>	<i>B. thetaiotaomicron</i>	<i>C. sputorum</i>	<i>S. aureus</i>	<i>E. coli</i>
1 × 10 ⁻⁵	+++	+++	+++	+++	+++	+++	+++	+++
5 × 10 ⁻⁵	+++	+++	+++	+++	+++	+++	+++	+++
1 × 10 ⁻⁴	+++	+++	+++	+++	+++	++	+++	+++
2 × 10 ⁻⁴	+++	+++	+++	+++	-	-	+++	+++
3 × 10 ⁻⁴	+++	+++	+++	+++	-	-	+++	+++
4 × 10 ⁻⁴	+++	+++	+++	+++	-	-	+++	+++
5 × 10 ⁻⁴	+++	+++	+++	+++	-	-	-	+
6 × 10 ⁻⁴	+++	+++	+++	+++	-	-	-	-
7 × 10 ⁻⁴	+++	+++	+++	+++	-	-	-	-
8 × 10 ⁻⁴	+++	+++	+++	+++	-	-	-	-
9 × 10 ⁻⁴	+++	+++	+++	+++	-	-	-	-
1 × 10 ⁻³	+++	+++	+++	+++	-	-	-	-
5 × 10 ⁻³	++	-	+++	+++	-	-	-	-
1 × 10 ⁻²	+	-	-	-	-	-	-	-
Control	+++	+++	+++	+++	+++	+++	+++	+++

Colony density on the plates was scored as follows: + + +, dense growth; + +, density reduced compared to control, but too numerous to count; +, number of colonies can be counted; -, no colonies.

Table 3
Effect of *Galla chinensis* extract on bacterial strains.

Samples	<i>E. faecalis</i>	<i>S. thermophilus</i>	<i>L. casei</i>	<i>B. longum</i>	<i>B. thetaiotaomicron</i>	<i>C. sputorum</i>	<i>S. aureus</i> (CFU/mL)	<i>E. coli</i> (CFU/mL)
H ₂ O ₂ (6.0 × 10 ⁻⁴ mol/L)	+++	+++	+++	+++	-	-	-	-
<i>Galla chinensis</i>	+++	-	-	-	-	-	53	0
<i>Galla chinensis</i> + catalase	+++	+	++	+	+++	++	3.8 × 10 ⁵	3.2 × 10 ⁴
Control	+++	+++	+++	+++	+++	+++	1.2 × 10 ⁵	1.5 × 10 ⁵
Control + catalase	+++	+++	+++	+++	+++	+++	1.3 × 10 ⁵	1.8 × 10 ⁶

Colony density on the plates was scored as follows: + + +, dense growth; + +, density reduced compared to control, but too numerous to count; +, the number of colonies can be counted; -, no colonies.

E. coli and *S. aureus* were shown the number of viable cells.

(*Trichophyton*) catechin, with associated degeneration of bacterial organelles, outflow of bacterial cytoplasm, and finally ghosting of the bacterial cells [13]. *Galla chinensis* is composed of gallotannin and further tannin hydrolysis products. Antimicrobial activity might be induced by these components acting on bacterial cell membranes in a manner similar to that of catechin. Similar to the results of our previous study [4–6], we revealed that hydrogen peroxide generated from *Galla chinensis* contributes to the antibacterial activity.

4. Conclusions

Hydrogen peroxide was generated from extracts of natural medicines *Galla chinensis*, *Gambir*, *Myrica cortex*, *Geranii herba*, *Malloti cortex*, and *Cinnamomi cortex* from four different sources. We demonstrated that the hydrogen peroxide generated from *Galla chinensis* contributed to the antibacterial activity of this natural product.

References

- [1] T. Okada, T. Suzuki, S. Hasobe, et al., Studies on bergenin (Report I), *Folia Pharmacol. Jpn.* 69 (1973) 369–378.

- [2] H. Takase, K. Yamamoto, K. Ito, et al., Pharmacological studies on antidiarrheal effects of berberine and *geranii herba*, *Folia Pharmacol. Jpn.* 102 (1993) 101–112.
- [3] T. Sawada, J. Yamahara, K. Goto, et al., Studies on evaluation of crude drug by bioassay. IV. Antibacterial activities of the components of *coptidis rhizoma*, *J. Pharm. Soc. Jpn.* 25 (1971) 74–78.
- [4] H. Arakawa, M. Kanemitsu, N. Tajima, et al., Chemiluminescence assay for catechin based on generation of hydrogen peroxide in basic solution, *Anal. Chim. Acta* 472 (2002) 75–82.
- [5] H. Arakawa, M. Maeda, S. Okubo, et al., Role of hydrogen peroxide in bactericidal action of catechin, *Biol. Pharm. Bull.* 27 (2004) 277–281.
- [6] H. Arakawa, M. Takasaki, N. Tajima, et al., Antibacterial activities of persimmon extract relate with their hydrogen peroxide concentration, *Biol. Pharm. Bull.* 37 (2014) 1119–1123.
- [7] Y.J. Ahn, C.O. Lee, J.H. Kweon, et al., Growth-inhibitory effects of galla rhois-derived tannins on intestinal bacteria, *J. Appl. Microbiol.* 84 (1998) 439–443.
- [8] Y. Maekawa, A. Taniguchi, T. Fukui, et al., Effect of tea catechins on serum vitamins, minerals and intestinal microflora, *Trace Nutr. Res.* 22 (2005) 93–99.
- [9] S. Okubo, T. Sasaki, Y. Hara, et al., Bactericidal and anti-toxin activities of catechin in enterohemorrhagic *Escherichia coli*, *J. Jpn. Assoc. Infect. Dis.* 72 (1998) 211–217.
- [10] Y. Horiuchi, M. Toda, S. Okubo, et al., Protective activity of tea and catechins against *Bordetella pertussis*, *J. Jpn. Assoc. Infect. Dis.* 66 (1992) 599–605.
- [11] O. Djakpo, W. Yao, *Rhus chinensis* and *galla chinensis* – Folklore to modern evidence: review, *Phytother. Res.* 24 (2010) 1739–1747.
- [12] H. Ohashi, K. Arita, Studies on the determination of tannin in crude drugs in Japanese pharmacopoeia. I. determination of tannin in *Geranium nepalense* Sweet (add. seasonal and individual variation), *J. Pharm. Soc. Jpn.* 82 (1962) 82–86.
- [13] Y. Toyoshima, S. Okubo, M. Toda, et al., Effect of catechin on the ultrastructure of *Trichophyton mentagrophytes*, *J. Jpn. Assoc. Infect. Dis.* 68 (1994) 295–303.