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Antimicrobial activity of catechol isolated from *Diospyros kaki* Thunb. roots and its derivatives toward intestinal bacteria

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ABSTRACT

The growth-inhibiting activities of the methanol extract of *Diospyros kaki* Thunb. roots were examined on the growth of *Bifidobacterium breve*, *B. longum*, *Clostridium difficile*, *Clostridium perfringes*, *Escherichia coli*, and *Lactobacillus casei*. In addition, the biologically active component of *D. kaki* roots was purified using silica gel column chromatography and HPLC. The active component was characterised as catechol by spectroscopic analyses. The antimicrobial activity of the isolated catechol varied according to the dose and bacterial strains tested. Catechol significantly (++++) inhibited the growth of *C. perfringens* at 2.0 mg/disc, and moderately (++) inhibited its growth at 0.25 mg/disc. At a dose of 5.0 mg/disc, catechol significantly inhibited the growth of *C. difficile* and moderately inhibited the growth of *E. coli*. However, this isolate did not inhibit the growth of bifidobacteria and lactobacilli. When various functional groups were added to the catechol, selective growth-inhibiting activity against harmful intestinal bacteria was observed in response to treatment with low concentrations. Taken together, these findings indicate that *D. kaki* root-isolated catechol and its derivatives (4-nitrocatechol, 4-tert-butylcatechol, tetrabromocatechol) could be useful as preventive agents against diseases caused by harmful intestinal bacteria.

1. Introduction

Intestinal microorganisms that reside in the gastrointestinal tract form a highly complex bacterial community in which obligate anaerobes are dominant (Tannock, 2002). It is well known that as many as 500 bacterial species can inhabit the intestinal tract, although it is likely that only 40 species predominate in each human (Santoyo et al., 2005). These types of bacterial flora are in a dynamic equilibrium that may be altered by diet, medication, stress, ageing, and other various environmental factors (Shin & Ustunol, 2005). In addition, these organisms influence physiological function, drug efficacy, carcinogenesis, and immunological responses in the host. The factors contributing to the harmful effects of these organisms include *N*-nitroso compounds and aromatic steroids that biotransform a variety of ingested or endogenously formed compounds into potentially harmful metabolites (Hentaes, 1983; Misuoka, 1984; Modler, McKellar, & Yaguchi, 1990; Moore & Holdeman, 1974; Rood, McClane, Songer, & Tithall, 1997). The bacterial communities found in the intestines of gastric cancer, Crohn's disease (CD), and ulcerative colitis (UC) patients generally contain high concentrations of harmful bacteria such as Clostridia and eubacteria, and contain few lactic acid bacteria (LAB) (Arlette & Colombel, 2008; Lim, Jeon, Jeong, Lee, & Lee, 2007). The incidence of gastrointestinal diseases worldwide has increased over the last few years; therefore, there are a large number of patients with gastrointestinal diseases that are being administered prolonged courses of antibiotics (Arlette & Colombel, 2008). However, the repeated use of antibiotics can result in problems in humans including resistance and adverse effects on non-target organisms. In this regard, natural products have generated a high degree of interest due to their low level of side effects and toxicity, as well as their availability when compared to currently available antibiotics (Vagionas et al., 2007).

Studies conducted to address serious problems that are often associated with antibiotics often focus on the isolation and characterisation of novel antimicrobial compounds from a variety of sources, including medicinal plants. The fruits and leaves of *Diospyros kaki* have long been used in Korean traditional medicines to treat cough, apoplexy and arteriosclerosis, and as an antioxidant (An, Bae, & Choi, 1998; Özen, Colack, Dincer, & Gűner, 2004). *D. kaki* is rich in antioxidant phenolic compounds other than tannins, and may reduce the risk of chronic diseases (Gorinstein et al., 1994; Özen et al., 2004). However, relatively little work has been conducted to evaluate the effects of *D. kaki* root-derived phenolic materials on the growth of intestinal microorganisms in spite of its excellent pharmacological effects. In this study, the antimicrobial activities of *D. kaki* root-derived materials against intestinal bacteria were determined. In addition, we evaluated the active principle isolated from *D. kaki* roots by spectroscopic analyses.

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Moreover, we evaluated the antimicrobial activities of derivatives of this isolate against intestinal bacteria.

2. Materials and methods

2.1. Chemicals

4-Chlorocatechol, 3-methoxycatechol, 3-methylcatechol, 4-methylcatechol, 4-nitrocatechol, 4-tert-butylcatechol, and tetrabromocatechol were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals used in this study were of reagent grade.

2.2. Isolation and identification of the compound

The roots of *D. kaki* were collected during the spring of 2006 in Chonbuk Province, Korea. The samples (5 kg) were washed three times with distilled water and dried in an oven at 40 °C for 2 days and finely powdered. The roots were extracted twice with methanol (2 × 10 l) at room temperature for 2 days. The extract was applied to filtration through filter paper (Toyo filter paper No. 2, Toyo Roshi, Tokyo, Japan) *in vacuo*. The combined filtrates were then concentrated *in vacuo* at 45 °C using a rotary vacuum evaporator (Model: N-3NW, EYELA, Japan). The extract (150 g) was then sequentially partitioned into hexane (2.79 g), chloroform (30.0 g), ethyl acetate (43.8 g), butanol (23.2 g), and water-soluble (50.2 g) portions for subsequent bioassays with intestinal bacteria. To purify the active compound, the chloroform portion was chromatographed on a silica gel column (Merck 70-230 mesh; 600 g; inside diameter [i.d.] 5.5 by 68 cm, Rahway, NJ, USA) and successively eluted with a gradient step of chloroform:methanol (3:7–0:10, v/v) to give six fractions (C1 to C6). The C5 fraction showed the most growth-inhibiting activity against *Clostridium difficile*, *C. perfringens*, and *Escherichia coli*. To purify the active C5 (1.31 g) fraction, it was subjected to preparative HPLC (Recycling Preparative HPLC, Japan Analytical Industry Co., LTD, Tokyo, Japan) to separate the biologically active constituent, after which the eluates were examined for their growth-inhibiting activities. The first column used was a Jaigel W Series Column (W-253 50 cm plus W-252 50 cm, 20.0 mm i.d., 1000 mm long; Japan Analytical Industry Co., LTD, Japan), using methanol (100%, v/v) at a flow rate of 5 ml/min and detection at 280 nm. The active C5 sample was subjected to this procedure two consecutive times, which resulted in isolation of an active compound (C511, 97 mg). The structure of the active isolate was determined by the instrumental analyses. IR spectra were recorded on ThermoNicolet/NEXus 870 FT-IR. Spectra were run in the 4000–400 cm^{-1} region. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform using a JNM-LA 400F7 spectrometer at 400 and 100 MHz (TMS as an internal standard), respectively, and the chemical shifts were given in δ (parts per million). Unambiguous ^{1}H and ^{13}C NMR chemical shifts were determined based on ^{1}H–^{13}H DEPT spectrum as well as the ^{1}H–^{13}H COSY spectrum. Specifically, this compound was identified on the basis of the following evidence: catechol ($C_6H_6O_2$, MW: 110.11); EI-MS (70 eV) *m*/*z* (% relative intensity) M^+ 110, 95, 69, 53; FT-IR (wavenumber, cm^{-1}); 849 (=CH), 1514–1620 (C=C), 1923 (benzene ring), 3052 (C—H), 3326–3450 (OH); ^{1}H NMR (CD_3OD, 400 MHz); δ 6.85–6.87 (1H, *m*), 6.79–6.82 (1H, *m*), 5.00–5.35 (OH, *d*); ^{13}C NMR (CD_3OD, 100 MHz); δ 143.67, 121.09, 115.41. In addition, a UV spectra was obtained in chloroform using a Jasco V-550 spectrometer.

2.3. Gas chromatography–mass spectrometry (GC–MS)

D. Kaki root was analysed on a gas chromatograph (6890, Agilent)-mass spectrometer (5973 IV, Agilent) (GC–MS). A 30 m × 0.25 mm inside diameter DB-5 (0.25 mm film) fused silica capillary column (J&W Scientific, Folsom, CA, USA) was used as the GC column. The GC conditions were as follows: injector temperature, 210 °C; column temperature, isothermal at 50 °C for 15 min, then programmed to rise to 200 °C at 2 °C/min and held at this temperature for 15 min; ion source temperature, 230 °C. Helium was used as the carrier gas at a rate of 0.8 ml/min. The effluent of the GC column was introduced directly into the source of the mass spectrometer. Spectra were obtained in the EI mode with 70 eV ionisation energy. The sector mass analyser was set to scan from 50 to 600 amu for 2 s. Compounds were identified by comparison with retention times and the mass spectra obtained with the authentic standards on the GC–MS system used for analysis. When an authentic sample was not available, identification for caryophyllene oxide was carried out by comparison of mass spectra obtained experimentally with those in the mass spectra library (The Wiley Registry of Mass Spectral Data, 6th Ed.).

2.4. Bacterial strains and culture conditions

The intestinal bacteria used in this study were *Bifidobacterium breve* ATCC 15700, *B. longum* ATCC 15707, *C. difficile* ATCC 9689, *C. perfringens* ATCC 13124, *E. coli* ATCC 11775, and *Lactobacillus casei* ATCC 393. Stock cultures of these strains were routinely stored on Eggerth-Gagnon (EG) liver extract-Field's slants at −80 °C and subcultured on EG agar (Eiken chemical, Tokyo, Japan) when required. The plates were incubated anaerobically at 37 °C for 2 days in an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2 in an anaerobic chamber (Hirayama, Tokyo, Japan). The bacteria were then grown in a Brain Heart Infusion broth (pH 7.6) and Microbiology Rogosa and Sharpe broth (pH 5.7).

2.5. Antimicrobial activity

The paper disc agar diffusion method was used to assess the antimicrobial activities of the test samples. To assay the effect of the test materials on the growth of the test microorganisms, one loopful of bacteria was suspended in 1 ml of sterilized physiological saline. An aliquot (0.1 ml) of the bacterial suspensions was then seeded onto the EG agar. A sample in 0.1 ml of methanol solution was then applied to a paper disc using a Drummond glass microcapillary (Advantec, diameter 8 mm and thickness 1 mm, Toyo Roshi, Japan). After the solvent evaporated, the discs were placed on the surface of the agar that had been inoculated with the test bacteria. All plates were then incubated anaerobically at 37 °C for 2 days. Control discs received 0.1 ml of methanol. All tests of growth inhibition were replicated three times. The antimicrobial activity was then determined by assigning one of the following values based on the estimated size (diameter) of the zone of inhibition produced by the test compounds: potent response (++++), zone of inhibition diameter >30 mm; strong response (+++), zone of inhibition diameter 21–30 mm; moderate response (++), zone of inhibition diameter 16–20 mm; weak response (+), zone of inhibition diameter 10–15 mm; and little or no response (−), zone of inhibition diameter <10 mm.

3. Results and discussion

The growth-inhibiting effects of the methanol extracts of *D. kaki* roots were assayed using the paper disc agar diffusion method against six intestinal bacteria. During routine screening, treatment with the methanol extracts at a concentration of 5.0 mg/disc exhibited strong inhibiting activity (+++) against *C. perfringens* and moderate (++) inhibition of *C. difficile* and *E. coli* (Table 1). However, the methanol extracts exerted no inhibitory response against *B. breve*, *B. longum*, and *L. casei*. Due to the potent activity of the

Table 1
Growth inhibitory responses of various fractions obtained from the methanol extract of Korean persimmon roots against intestinal bacteria.

Fraction[a]	Bacterial species[b]					
	B. adolescentis	*B. longum*	*C. difficile*	*C. perfringens*	*E. coli*	*L. casei*
Methanol fraction	–[c]	–	++	+++	++	–
Hexane fraction	–	–	–	–	–	–
Chloroform fraction	–	–	++	+++	++	–
Ethyl acetate fraction	–	–	–	–	–	–
Butanol fraction	–	–	–	–	–	–
Water fraction	–	–	–	–	–	–

[a] Exposed to 5 mg/disc.
[b] Cultured on Eggerth-Gagnon agar at 37 °C for 2 days under an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2.
[c] Zone of inhibition diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –.

methanol extract derived from *D. kaki* roots, isolation of the active component was pursued. To accomplish this, bioassay-guided fractionation of the methanol extracts was conducted. The results of these assays revealed significant differences in the growth-inhibiting activity of the fractions against the intestinal bacteria tested. Specifically, at a dose of 5.0 mg/disc, the chloroform fraction showed strong (+++) inhibitory activity against *C. perfringens*, moderate inhibitory against *C. difficile* and *E. coli*, and no adverse effects on the growth of beneficial bacteria. However, no activity was observed when the hexane, ethyl acetate, butanol, and water fractions were tested against intestinal bacteria. Therefore, the growth-inhibiting constituent of the chloroform fraction was isolated by silica gel column chromatography and HPLC. Structural determination of the isolate was made by spectroscopic analyses including UV, EI-MS, ^1H–^{13}H COSY, ^1H–^{13}H DEPT, ^{13}C NMR and ^1H NMR, comparing directly with authentic reference compounds. Based on the results of these analyses, the biologically active constituent was identified as catechol (C511). The spectroscopic data

Table 2
Growth inhibiting responses of catechol and its derivatives against intestinal bacteria[a].

Compound	Dosage (mg/disc)	Bacterial strain[b]					
		B. breve	*B. longum*	*C.difficile*	*C. perfringens*	*E. coli*	*L. casei*
Catechol	5.0	–[c]	–	++++	++++	++	–
	2.0	–	–	++	++++	+	–
	1.0	–	–	++	+++	–	–
	0.5	–	–	+	++	–	–
	0.25	–	–	–	++	–	–
	0.1	–	–	–	+	–	–
4-Chlorocatechol	5.0	+++	+++	+++	+++	+++	+++
	2.0	++	++	++	++	++	++
	1.0	++	–	++	++	–	–
	0.5	+	–	–	–	–	–
3-Methoxycatechol	5.0	–	+	+	–	+	–
	2.0	–	–	–	–	–	–
	1.0	–	–	–	–	–	–
3-Methylcatechol	5.0	–	+	++	–	++	–
	2.0	–	–	+	–	+	–
	1.0	–	–	–	–	–	–
4-Methylcatechol	5.0	–	+	++	–	+	–
	2.0	–	+	+	–	+	–
	1.0	–	–	–	–	–	–
4-Nitrocatechol	5.0	+++	++++	+++	+++	+++	+++
	2.0	+++	++++	+++	+++	+++	+++
	1.0	++	++	++	++	++	+
	0.5	–	–	+	++	–	–
	0.25	–	–	–	+	–	–
4-Tert-butylcatechol	5.0	+++	+++	+++	+++	++++	+++
	2.0	++	++	++	+++	++	+++
	1.0	–	–	+	++	++	+
	0.5	–	–	–	+	+	–
	0.25	–	–	–	–	–	–
Tetrabromocatechol	5.0	+++	–	+++	++++	++++	++++
	2.0	+++	–	++	+++	++++	+++
	1.0	++	–	++	++	+++	+++
	0.5	+	–	+	++	+++	++
	0.25	–	–	–	+	+++	++
	0.10	–	–	–	–	+	+
Tetracycline	5.0	++++	++++	++++	++++	++++	++++
	2.0	++++	++++	++++	++++	++++	++++
	1.0	++++	++++	++++	++++	++++	++++
	0.5	++++	++++	++++	++++	+++	++++
	0.25	+++	++++	++++	++++	+++	++++

[a] Each assay was conducted in triplicate.
[b] Cultured on Eggerth-Gagnon agar at 37 °C for 2 days under an atmosphere comprised of 80% N_2, 15% CO_2, and 5% H_2.
[c] Zone of inhibition diameter >30 mm, ++++; 21–30 mm, +++; 16–20 mm, ++; 10–15 mm, +; and <10 mm, –.

generated by the analysis of catechol in this study matched those of previously reported catechol (Hamzah & Tu, 1981).

The growth-inhibiting activity of catechol against the six intestinal bacteria was examined using the paper disc agar diffusion method (Table 2). Treatment with catechol at a concentration of 5.0 mg/disc was found to significantly inhibit the growth of *C. difficile* and *C. perfringens*, and to moderately inhibit the growth of *E. coli*. However, treatment with this concentration of catechol had no effect on the growth of *B. breve*, *B. longum*, and *L. casei*. Furthermore, this isolate strongly inhibited the growth of *C. perfringens* at a concentration of 2.0 and 1.0 mg/disc, and moderately inhibited the growth of *C. perfringens* at 0.5 and 0.25 mg/disc. Catechol also exerted moderate inhibition against *C. difficile* at 1.0 mg/disc and weak activity against *E. coli* at 2.0 mg/disc. The growth-inhibiting activity of catechol against six intestinal bacteria was then compared to that of catechol derivatives of the commercially available antibiotic, tetracycline (Table 2, Fig. 1). When the effects of the derivatives on the growth of *C. difficile* and *E. coli* were evaluated, treatment with 3-methoxycatechol, 3-methylcatechol, and 4-methylcatechol at a concentration of 5.0 mg/disc resulted in moderate or weak inhibition. In addition, these compounds exerted no adverse effects on the growth of *B. breve*, *C. perfringens*, and *L. casei*. Treatment with 4-chlorocatechol, 4-nitrocatechol, 4-tert-butylcatechol, and tetrabromocatechol at a concentration of 5.0 mg/disc strongly inhibited the growth of Clostridia and *E. coli*.

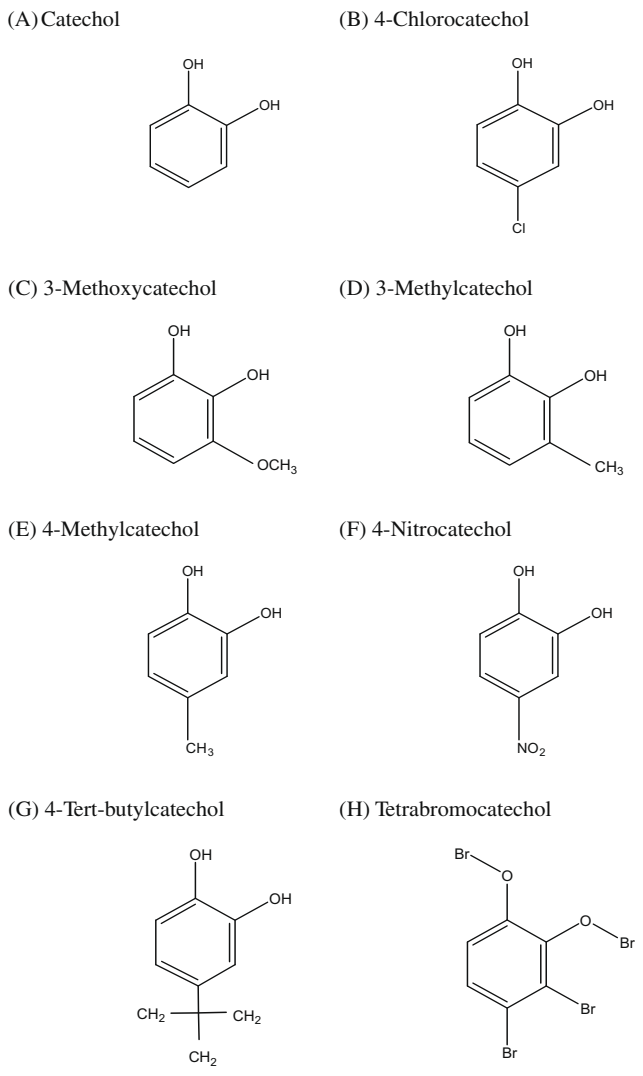

Fig. 1. Structure of catechol and its derivatives.

In addition, treatment with 4-chlorocatechol at a concentration of 2.0 and 1.0 mg/disc exerted moderate growth inhibition against Clostridia and treatment with 2.0 mg/disc exerted moderate growth inhibition against *E. coli*. Treatment with 4-nitrocatechol, 4-tert-butylcatechol, and tetrabromocatechol at a concentration of 2.0 mg/disc strongly inhibited the growth of *C. perfringens*, whereas treatment with 1.0 and 0.5 mg/disc moderately inhibited its growth. Tetrabromocatechol exhibited strong growth inhibition against *E. coli* at concentrations of 2.0, 1.0, 0.5, and 0.25 mg/disc, whereas it exerted no growth inhibition against *B. longum* at concentrations of 5.0 and 2.0 mg/disc. In addition, even though 4-nitrocatechol and 4-tert-butylcatechol exerted moderate or no growth inhibition against *B. breve*, *B. longum*, and *L. casei* at 1.0 mg/disc, these compounds showed no growth inhibition against beneficial bacteria at low concentrations. However, treatment with tetracycline resulted in very strong and strong growth inhibition of intestinal bacteria at doses as low as 0.5 and 0.25 mg/disc. Taken together, these results indicate that the growth-inhibiting activity of catechol was more pronounced in *C. difficile*, *C. perfringens*, and *E. coli* than in bifidobacteria and *L. casei*. It is highly desirable to inhibit the growth of harmful bacteria without exerting adverse effects on beneficial bacteria in the human intestinal tract.

Many studies have been conducted to evaluate the effects of selective growth promoting factors and selective growth-inhibiting factors against intestinal bacteria. For example, Lim et al. reported that methanol extract of *Caesulpinia sappan* inhibited the growth of *C. perfringens* amongst intestinal harmful bacteria (Lim et al., 2007). Furthermore, extracts from green tea (*Thea sinensis* L.) have been shown to enhance the growth of bifidobacteria and to inhibit the growth of Clostridia (Ahn, Kawamura, Kim, Yamamoto, & Mistuoka, 2005; Park et al., 2005). Due to increasing development of drug resistance to human pathogenic organisms, as well as the undesirable side effects of certain antibiotics, there is a pressing need for the development of safer treatments (Radulovic et al., 2007).

D. kaki is rich in antioxidant phenolic compounds, such as catechin and catechol (Ahn et al., 2002; Özen et al., 2004). In this study, the extract of *D. kaki* roots was found to inhibit the growth of *C. difficile*, *C. perfringnes*, and *E. coli*. In addition, the inhibitory activity of catechol isolated from *D. kaki* confirmed that it effectively exerts selective activity against intestinal bacteria. Specifically, these compounds exerted potent growth-inhibiting activity against Clostridia and *E. coli*, but no growth inhibition against beneficial bacteria. Furthermore, when various functional groups were added to catechol, they were found to exert selective growth-inhibiting activity against harmful intestinal bacteria at low concentrations. Interestingly, catechol structures substituted at the C-4 position with a functional group, including 4-chlorocatechol, 4-nitrocatechol, and 4-tert-butylcatechol, were more effective toward intestinal bacteria than catechol structures substituted at the C-3 position, such as 3-methoxycatechol, and 3-methylcatechol. In particular, nitro, and tert-butyl substitution of the catechol backbone at the C-4 position leads to selective growth-inhibiting activities at low concentrations. However, tetracycline inhibited the growth of all bacteria tested at low concentrations.

This is the first study to report the selective antimicrobial activities of catechol and its derivatives against intestinal bacteria. The results of many previous studies have indicated that catechols act as antioxidants in eukaryotic cells, thereby preventing degenerative diseases such as cancer and heart disease (Berberian, Allen, Sharma, Boyd, & Hardacre, 2007). In biology, compounds containing catechol play an important role in diverse metabolic pathways (Robinson, Stephens, Dalton, & Geary, 1992; Sigma–Aldrich, 2006). The oral LD_{50} values of catechol for mice and mammals have been reported to be 260 and 240 mg/kg, respectively (Sigma–Aldrich, 2006).

In conclusion, the antimicrobial activity of catechol against harmful intestinal bacteria combined with almost no adverse effects on beneficial bacteria indicates that *D. kaki* roots may have pharmacological value. However, further work is necessary to determine if this activity is great enough to justify evaluating these antimicrobial compounds in clinical applications to determine if their use in humans is feasible.

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