Antibacterial Spectrum of Plant Polyphenols and Extracts Depending upon Hydroxyphenyl Structure

Toshitsugu TAGURI,*[,a,b] Takashi TANAKA,[b] and Isao KOUNO[b]

[a] *Nagasaki Prefectural Institute of Public Health and Environmental Sciences; 1–9–5 Nameshi, Nagasaki 852–8061, Japan: and* [b] *Course of Pharmaceutical Sciences, Graduate School of Biomedical Sciences, Nagasaki University; 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan.*

Received May 12, 2006; accepted August 8, 2006; published online August 16, 2006

The relationship between the structure and antibacterial activity of 22 polyphenols was analyzed by using minimum inhibitory concentration (MIC) as a criterion against 26 species of bacteria which can grow in Mueller–Hinton medium. There was no clear correlation between Gram-staining and bacterial susceptibility to polyphenols, and the extent of the susceptibility was approximately dependent on the species of bacteria. In the same Gram-negative bacteria, the antibacterial activity of the polyphenols against *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* was comparatively strong. On the other hand, the activity against 11 species of the Enterobacteriaceae was comparatively weak, and the activity against six species of aerobic bacteria causing plant disease was moderate. Polyphenols having pyrogallol groups showed strong antibacterial activity, and those with catechol and resorcinol rings showed lower activity. The structure–activity relationship was extended to 26 polyphenol-rich plant extracts which could have potent antibacterial activity suitable for commercial use.

Key words antibacterial activity; hydroxyphenyl group; minimum inhibitory concentration; polyphenol; pyrogallol group

Recently, many biochemical and epidemiological studies have revealed that polyphenols of various foods and herbs have benefits to human health, and some extracts of polyphenol-rich plants, such as green tea and grape seed, have been added to foods or supplements. The antibacterial activity of various plant polyphenols and plant extracts have also been evaluated in several pharmaceutical studies.[1,2] Although tea polyphenols and their extracts have been examined in detail,[3—6] other extracts of polyphenol-rich plants have not been properly evaluated, because the activity of their chemical constituents has not been clearly demonstrated.[7,8]

Many reports on the antibacterial activity of pure polyphenols have been published,[4,9—12] however, each result was not able to be compared directly because different methods of evaluation were applied and various bacterial species were used. In order to overcome this problem, we compared the activity of a wide variety of polyphenols against many bacterial species to clarify their antibacterial spectrum, using the same standard method of the minimum inhibitory concentration (MIC). As a result, we found a relatively simple structure–activity relationship, and it was applied to the plant extracts containing various types of polyphenols.

MATERIALS AND METHODS

Chemicals Twenty-two pure or partially pure polyphenols and 26 plant extracts were used in this study (Fig. 1). Polyphenols **1**—**10** were the same as in our previous study.[12] Epigallocatechin (EGC, **1**) and epigallocatechin-3-*O*-gallate (EGCg, **2**) were isolated from commercial green tea; punicalagin (**3**) was isolated from the peel of *Punica granatum*; tannic acid (**4**) was purchased from Kanto Chemical Co., Japan; castalagin (**5**) was isolated from the wood of *Castanea crenata*; and prodelphinidins (**6**) were isolated from the bark of *Elaeocarpus sylvestris* var. *ellipticus*; geraniin (**7**) was isolated from the leaves of *E. sylvestris* var. *ellipticus*; loquat procyanidins (**8**) were isolated from the seeds of *Eriobotrya japonica*; theaflavins (**9**) was obtained from black tea; and loquat-treated green tea polyphenols (**10**) were prepared by treatment of commercial green tea with unripe loquat fruit. Gallic acid monohydrate (**11**) was purchased from Wako Pure Chemical Industries, Japan. Thearubigin (**12**) was prepared from black tea as follows: aqueous acetone extract was successively partitioned with AcOEt and *n*-BuOH. The *n*-BuOH layer was concentrated and subjected to Sephadex LH-20 column chromatography. Elution of 50% acetone yielded **12**. (+)-Catechin (**13**) and (−)-epicatechin gallate (ECg, **14**) were isolated from gambir and green tea, respectively. Myricitrin (**15**) and rutin (**16**) were obtained from bark of *Myrica rubra* and flower bud of *Sophora japonica*, respectively. Theaflavin mixture (**17**) was separated from AcOEt soluble fractions of aqueous acetone extracts of black tea. Pyrocatechol (**18**), pyrogallol (**19**), protocatechuic acid (**20**), and caffeic acid (**21**) were purchased from Sigma Aldrich Japan. Resveratrol (**22**) was isolated from dried roots of *Polygonum cuspidatum*.

Twenty-six plant extracts (**23**)—(**48**) were prepared by extraction with hot water (100 °C for 1 h), respectively. After filtration, the filtrate was applied to a column of MCl-gel CHP20P (Mitsubishi Chemical Corporation, Japan). After washing the column with water, the polyphenols were eluted out with 30—80% MeOH. The plant extracts were classified into five groups according to their constituents. The fermented tea leaves (**23**) and fresh tea leaves (**24**) originated from *Camellia sinensis* and mainly contained catechin derivatives. Therefore, **23** and **24** were classified into the catechin group. *Bischofia javanica* (**25**), *E. sylvestris* var. *ellipticus* (**26**), *Sapium sebiferum* (**27**), *Camellia japonica* (**28**), *Cornus brachypoda* (**30**), *Fragaria grandiflora* (**32**) (flesh leaves), *Stachyurus praecox* (**29**) (unripe fruits), and *Castanea crenata* (**31**) (fresh bark) mainly contained ellagitannin; thus, these were classified into the ellagitannin group. *Fragaria grandiflora* (**35**) (fresh stalk), *Citrus unshiu* (**33**), *Liquidambar formosana* (**34**), and *Myrica rubra* (**36**) (fresh

* To whom correspondence should be addressed. e-mail: tagurit@pref.nagasaki.lg.jp

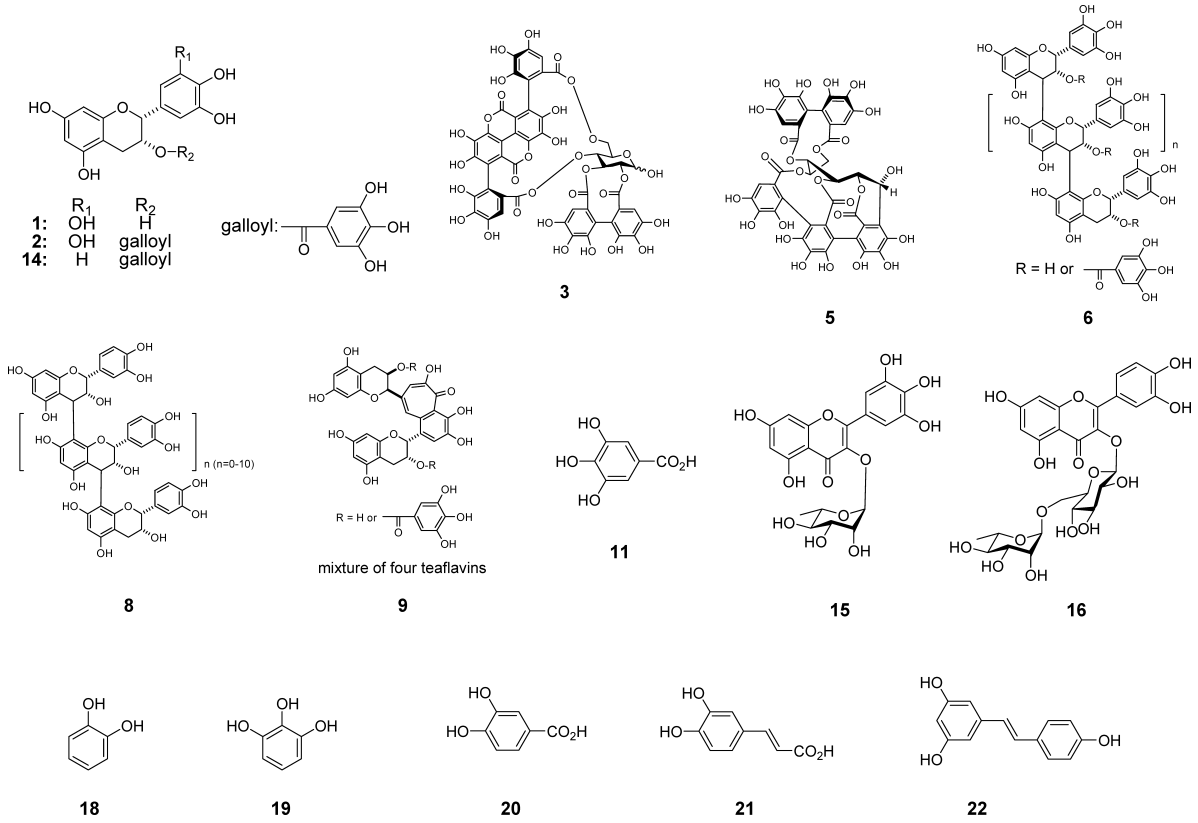

Fig. 1. Chemical Structures of Main Polyphenols Used in This Study

leaves) were classified into the flavonoid group because these extracts contained flavonoid glycosides as the major component. *Paeonia lactiflora* (**37**) (fruits), *Distylium racemosum* (**38**) (leaves), and *Castanopsis cuspidata* (**39**) (leaves) mainly contained gallotannin, therefore, these were classified into the gallotannin group. *Cryptomeria japonica* (**40**) (bark), *Pinus pinaster* (**41**) (bark), *Chamaecyparis obtuse* (**42**) (bark), *Acacia dealbata* (**43**) (fruits), *Cinnamonum camphora* (**44**) (leaves), *Diospyros kaki* (**45**) (leaves), *Eriobotrya japonica* (**46**) (leaves), *Pasania edulis* (**47**) (leaves), and *Vitis vinifera* (**48**) (unripe fruits) mainly contained procyanidins and these extracts were classified into the procyanidin group. After 160 mg freeze-dried powder of each sample was weighed, they were suspended in 5 ml sterile distilled water or 10% DMSO solution. Each specimen was heat-extracted and used as an undiluted solution.

Bacterial Strains Bacterial strains are listed in Table 1. In our previous study,[12] it was shown that polyphenols show stronger antibacterial activity against *Staphylococcus aureus* subsp. *aureus* (*S. aureus*), which is a kind of Gram-positive, than *Escherichia coli* and *Salmonella* spp., which are kinds of Gram-negative. Polyphenols also showed activity against the Gram-negative genus *Vibrio*. There is no clear correlation between Gram-staining and antibacterial activity of polyphenols, as described by some researchers.[5,13,14] In this study, we selected bacterial species on the basis of those results. *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens*, *Listeria monocytogenes*, *S. aureus* and *Clavibacter michiganensis* were used as Gram-positive bacteria. *C. perfringens* is an obligately anaerobic bacteria, which cannot grow in the presence of oxygen, *B. subtilis* and *C. michiganensis* are aerobic,

Table 1. Bacterial Strains Used in This Study

Bacteria	Source	Gram staining	Oxygen requirement
Bacillus cereus	ATCC[a] 11778	+	Facultatively anaerobic
Bacillus subtilis	ATCC 6633	+	Aerobic
Clostridium perfringens	ATCC 13124	+	Obligately anaerobic
Listeria monocytogenes	ATCC 7644	+	Facultatively anaerobic
Staphylococcus aureus	ATCC 29213	+	Facultatively anaerobic
Clavibacter michiganensis	MAFF[b] 301494	+	Aerobic
Aeromonas hydrophila	ATCC 7966	−	Facultatively anaerobic
Vibrio parahaemolyticus	ATCC 17802	−	Facultatively anaerobic
Vibrio vulnificus	ATCC 27562	−	Facultatively anaerobic
Citrobacter freundii	ATCC 8090	−	Facultatively anaerobic
Escherichia coli	ATCC 25922	−	Facultatively anaerobic
Klebsiella pneumoniae	ATCC 13883	−	Facultatively anaerobic
Proteus mirabilis	ATCC 7002	−	Facultatively anaerobic
Proteus vulgaris	ATCC 6380	−	Facultatively anaerobic
Salmonella Anatum	ATCC 9270	−	Facultatively anaerobic
Salmonella arizonae	ATCC 13314	−	Facultatively anaerobic
Shigella flexneri	ATCC 12022	−	Facultatively anaerobic
Shigella sonnei	ATCC 25931	−	Facultatively anaerobic
Yersinia enterocolitica	ATCC 9610	−	Facultatively anaerobic
Erwinia carotovora	MAFF 211382	−	Facultatively anaerobic
Pseudomonas aeruginosa	ATCC 27853	−	Aerobic
Pseudomonas cichorii	MAFF 311390	−	Aerobic
Pseudomonas marginalis	MAFF 302400	−	Aerobic
Pseudomonas viridiflava	MAFF 302660	−	Aerobic
Agrobacterium tumefaciens	MAFF 301001	−	Aerobic
Xanthomonas campestris	MAFF 301780	−	Aerobic

a) ATCC, American Type Culture Collection. *b*) MAFF, Ministry of Agriculture, Forestry and Fisheries.

Table 2. Comparison of Mean Minimum Inhibitory Concentration (MIC) of Purified Polyphenols and Plant Extracts for Different Bacterial Groups

Chemicals	Bacterial group			
	Gram-positive (Group I)	Facultatively anaerobic gram-negative (Group II)	Facultatively anaerobic gram-negative (Group III)	Aerobic gram-negative (Group IV)
Purified polyphenols	819±528*	316±265**	866±596*,$	690±507*,$$
Plant extracts	1152±501!	390±429!!	1227±494!,#	1001±499!,##

Values are given as mean±S.D. (μg/ml). *t*-Test showed significant differences between * and **, $ and $$, ! and !!, and # and ## ($p<0.01$).

and the others are facultatively anaerobic bacteria, which can be grow despite the presence of oxygen. *B. cereus*, *B. subtilis* and *C. perfringens* form spores which have higher structural durability. We mainly used the Vibrionaceae and the Enterobacteriaceae as facultatively anaerobic, Gram-negative bacteria. *Vibrio parahaemolyticus* and *Vibrio vulnificus* were selected from the family Vibrionaceae. *Aeromonas hydrophila* was added to these species because it had formerly belonged to the same family of them. Whereas *Citrobacter freundii*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Salmonella enterica* serovar Anatum (*S.* Anatum), *Salmonella enterica* subsp. *arizonae* (*S. arizonae*), *Shigella flexneri*, *Shigella sonnei*, *Yersinia enterocolitica* and *Erwinia carotovora* subsp. *carotovora* (*E. carotovora*) were selected from the family Enterobacteriaceae. Besides these species, *Pseudomonas aeruginosa*, *Pseudomonas cichorii*, *Pseudomonas marginalis*, *Pseudomonas viridiflava*, *Agrobacterium tumefaciens* and *Xanthomonas campestris*, which are Gram-negative aerobes were examined. *C. michiganensis*, *E. carotovora*, *P. cichorii*, *P. marginalis*, *P. viridiflava*, *A. tumefaciens* and *X. campestris* are pathogenic for some plants, and these were gifted from the National Institute of Agrobiological Sciences, Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan. Another 20 strains from the American Type Culture Collection (ATCC) were purchased from Amco, Japan.

Antimicrobial Activity Test The antimicrobial activity test was also followed from our previous study.[12] Briefly, for each polyphenol used, Mueller–Hinton agar plates were prepared with a final polyphenol concentration of 3200 to 25 μg/ml by the twofold serial dilution method. Bacterial strains were adjusted to approximately 10^6 CFU/ml in Mueller–Hinton broth and each bacterial inoculum was applied to the test medium using a 1-μl disposable inoculation loop (Aktiengesellschaft, Germany) in approximately 2-cm streaks. The test culture medium inoculated with *C. perfringens* was inserted into an anaerobic box with an anaerobic pack (Mitsubishi Gas Chemical Company, Japan) and incubated at 36±1 °C for 19±1 h. Seven species of plant disease-causing bacteria were incubated in aerobic conditions at 30±1 °C for 19±1 h. The other bacteria were incubated in aerobic conditions at 36±1 °C for 19±1 h. The minimum concentration without bacterial growth was judged to be the MIC and the mean value for each polyphenol or extract against each bacterium was calculated from three independent experiments. The criterion for the strength of antibacterial activity was taken as the original standard value of 800 μg/ml, established by the Society of Industrial-Technology for Antimicrobial Articles (SIAA), which was agreed by various organizations, including antibacterial agent manufacturers, product manufacturers, and the testing laboratory of Japan in 1998.[16] We adapted this standard value as the criterion for the MIC, and defined strong activity for <400 μg/ml, moderate activity for 400—800 μg/ml, and weak activity for >800 μg/ml.

Statistical Processing The Student's *t*-test was performed to analyze the differences between the mean MICs of the four aforementioned bacterial groups.

RESULTS

Antibacterial Spectrum of Purified Polyphenols The bacterial species were classified into four groups based on Gram-staining and oxygen requirements. Group I comprised Gram-positive bacteria. Group II comprised *A. hydrophila* and two species of the Vibrionaceae. The Enterobacteriaceae and the Gram-negative aerobic bacteria were classified into groups III and IV, respectively. Exceptionally, *C. michiganensis* was classified into group I because it is Gram-positive despite its aerobic properties.

The susceptibility (mean MIC±S.D., μg/ml) to polyphenols was significantly different for each group of bacteria (Table 2): the group II, which mainly consists of the Vibrionaceae, showed strong susceptibility (316±265); the group IV aerobic bacteria showed moderate susceptibility (690±507); and the group I Gram-positive bacteria (819±528) and the group III Enterobacteriaceae (866±596) showed considerably weak susceptibility. There were significant differences between the MIC values in group II and the other groups, and between the MICs of group III and group IV ($p<0.01$).

The susceptibility (mean MIC±S.D., μg/ml) of each bacterial species to purified polyphenols is shown in Table 3. *C. perfringens* (143±108) (group I), *V. parahaemolyticus* (214±175) and *V. vulnificus* (299±261) (group II) had high susceptibility. In contrast, *B. cereus* (860±771), *B. subtilis* (1382±852), *L. monocytogenes* (1297±896) (group I), *C. freundii* (817±541), *E. coli* (1364±919), *S.* Anatum (1211±914), *S. arizonae* (977±730), *S. flexneri* (820±653), *S. sonnei* (1023±783), *Y. enterocolitica* (802±638 μg/ml) (group III), *P. aeruginosa* (997±740) and *P. marginalis* (846±781) (group IV) showed weak susceptibility. *S. aureus* (491±484), *C. michiganensis* (463±382) (group I), *A. hydrophila* (435±465) (group II), *K. pneumoniae* (561±676), *P. mirabilis* (600±295), *P. vulgaris* (537±323), *E. carotovora* (498±409) (group III), *P. cichorii* (572±513), *P. viridiflava* (591±587), *A. tumefaciens* (577±411), and *X. campestris* (556±485) (group IV) showed moderate susceptibility.

Antibacterial Activity of Purified Polyphenols The antibacterial activity (mean MIC±S.D., μg/ml) of each polyphenol was compared (Table 3). EGC (**1**) (360±184),

Table 3. Mean Minimum Inhibitory Concentration (MIC) in Each Purified Polyphenol against Each Bacterial Species

Bacterial group[a)]		MIC (μg/ml) in purified polyphenols												
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Group I	*Bacillus cereus*	400	267	667	533	467	667	800	467	933	800	1067	533	3200
	Bacillus subtilis	933	533	467	667	600	933	1333	1867	2133	2133	1600	2666	2667
	Clostridium perfringens	233	50	67	50	67	67	67	233	133	133	83	167	433
	Listeria monocytogenes	400	400	3200	1600	667	1067	933	2133	1600	2667	1600	1333	1067
	Staphylococcus aureus	167	133	600	400	267	267	333	267	467	600	533	400	533
	Clavibacter michiganensis	333	200	133	117	233	533	267	800	1333	1067	333	800	533
	Mean MIC±S.D.	411±472	264±178	856±1174	561±561	383±233	589±383	622±480	961±834	1100±740	1233±969	869±652	983±916	1406±1216
Group II	*Aeromonas hydrophila*	117	83	400	200	150	300	200	667	467	1000	133	1200	100
	Vibrio parahaemolyticus	133	83	83	75	117	100	83	467	333	533	50	333	233
	Vibrio vulnificus	200	67	133	133	167	233	200	1067	333	533	67	467	267
	Mean MIC±S.D.	150±44	78±10	206±170	136±63	144±25	211±102	161±67	733±306	378±77	689±269	83±44	667±467	200±88
Group III	*Citrobacter freundii*	333	267	1067	667	333	667	400	1333	1200	800	600	800	1333
	Escherichia coli	667	533	2133	1333	533	667	1333	2133	1600	1600	600	1333	2667
	Klebsiella pneumoniae	150	133	333	267	233	400	233	533	500	400	400	400	333
	Proteus mirabilis	533	400	533	333	400	400	333	667	1000	400	667	800	1333
	Proteus vulgaris	400	400	333	267	400	333	267	533	667	533	533	1333	667
	Salmonella Anatum	400	333	800	533	533	667	1067	2667	1200	1200	500	2666	2667
	Salmonella arizonae	333	267	800	667	400	800	667	1333	1200	1200	667	1333	2667
	Shigella flexneri	667	333	533	333	333	533	400	2133	800	800	267	600	1333
	Shigella sonnei	333	400	533	400	400	533	533	1333	1200	1600	500	800	2133
	Yersinia enterocolitica	400	333	400	467	400	533	667	1867	1333	1067	533	600	400
	Erwinia carotovora	167	100	333	267	200	467	267	533	533	1200	467	1200	533
	Mean MIC±S.D.	398±170	318±124	709±528	503±313	379±104	545±142	561±355	1370±754	1021±353	982±431	521±118	1079±623	1461±935
Group IV	*Pseudomonas aeruginosa*	400	400	533	400	400	667	533	1867	1067	1333	533	933	1333
	Pseudomonas cichorii	333	200	333	267	333	467	333	667	600	800	333	1067	167
	Pseudomonas marginalis	400	200	333	467	467	667	533	2667	2133	2667	267	933	1333
	Pseudomonas viridiflava	267	200	267	133	200	333	200	2667	933	1333	267	533	533
	Agrobacterium tumefaciens	333	167	333	333	233	467	667	1067	600	1200	533	1333	667
	Xanthomonas campestris	333	167	267	167	133	467	400	1867	1067	667	133	1067	467
	Mean MIC±S.D.	344±50	222±89	344±98	294±131	294±127	511±131	444±167	1800±816	1067±564	1333±711	344±160	978±262	750±481
Total	Mean MIC±S.D.	360±184	256±141	601±668	426±357	333±157	509±233	502±349	1301±797	976±522	1087±642	510±394	986±609	1138±947

Table 3. continued

	Bacterial group[a)]	MIC (μg/ml) in purified polyphenols									Total	MIC (μg/ml) in positive controls	
		14	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**		ABPC[c)]	KM[d)]
Group I	*Bacillus cereus*	200	400	1333	467	267	117	1600	1067	2667	860±771	1.56	3.12
	Bacillus subtilis	533	667	2133	800	533	267	2667	1600	2667	1382±852	0.02	1.56
	Clostridium perfringens	100	267	NT[b)]	NT	NT	NT	NT	NT	NT	143±108		
	Listeria monocytogenes	400	467	2667	400	267	67	2133	1333	2133	1297±896	0.39	1.56
	Staphylococcus aureus	133	200	1067	100	167	25	667	1333	2133	491±484	0.49	6.25
	Clavibacter michiganensis	200	267	800	133	133	150	25	600	1200	463±382	0.38	3.12
	Mean MIC±S.D.	261±169	378±72	1600±777	380±284	273±157	125±92	1418±1073	1187±378	2160±599			
Group II	*Aeromonas hydrophila*	150	300	400	100	100	33	533	1067	1867	435±465	200	9.38
	Vibrio parahaemolyticus	67	167	533	67	83	25	533	267	333	214±175	1.56	12.50
	Vibrio vulnificus	83	267	667	67	67	25	667	333	533	299±261	12.50	6.25
	Mean MIC±S.D.	100±44	244±69	533±133	78±19	83±17	28±5	578±77	556±444	911±834			
Group III	*Citrobacter freundii*	467	533	1867	400	267	100	1333	1067	2133	817±541	12.50	3.12
	Escherichia coli	533	667	2133	400	533	83	2667	2667	3200	1364±919	6.25	9.38
	Klebsiella pneumoniae	267	267	667	133	267	25	2133	2667	1600	561±676	50	3.12
	Proteus mirabilis	533	533	1067	267	NT	NT	NT	NT	NT	600±295	3.12	3.12
	Proteus vulgaris	333	400	1333	400	NT	NT	NT	NT	NT	537±323		
	Salmonella Anatum	800	800	2133	400	267	67	2667	2133	2133	1211±914	3.91	6.25
	Salmonella arizonae	800	533	1067	333	200	83	2667	1333	2133	977±730	0.78	3.12
	Shigella flexneri	400	533	1600	333	467	42	1333	2133	2133	820±653	3.12	12.50
	Shigella sonnei	1067	1067	2133	400	167	42	2133	2133	2667	1023±783	2.34	12.50
	Yersinia enterocolitica	400	400	1600	333	267	33	1333	2133	2133	802±638		12.50
	Erwinia carotovora	333	333	933	100	100	400	100	1600	800	498±409	0.78	3.12
	Mean MIC±S.D.	539±248	552±227	1503±527	318±109	281±138	97±116	1819±867	1985±552	2104±659			
Group IV	*Pseudomonas aeruginosa*	667	800	2133	667	267	67	2133	2667	2133	997±740	200	200
	Pseudomonas cichorii	100	167	1200	200	200	25	1600	1600	1600	572±513	50	0.78
	Pseudomonas marginalis	200	400	1600	200	200	150	800	1200	800	846±781	200	3.12
	Pseudomonas viridiflava	267	467	800	200	200	200	800	1000	1200	591±587	100	0.78
	Agrobacterium tumefaciens	333	400	467	100	100	38	1333	800	1200	577±411	50	25
	Xanthomonas campestris	267	200	800	167	167	100	1333	1200	800	556±485	100	3.12
	Mean MIC±S.D.	306±194	406±227	1167±614	256±205	189±54	97±68	1333±506	1411±670	1289±511			
Total	Mean MIC±S.D.	371±252	442±220	1325±641	287±188	230±130	94±91	1443±840	1475±706	1748±768			

a) Each bacterial group is the same as in Table 2. *b*) Not tested; *c*) ABPC, ampicillin sodium; *d*) KM, kanamycin sulfate. Epigallocatechin (**1**), epigallocatechin-3-*O*-gallate (**2**), punicalagin (**3**), tannic acid (**4**), castalagin (**5**), prodelphinidins (**6**), geraniin (**7**), procyanidins (**8**), theaflavins (**9**), loquat-treated green tea polyphenols (**10**), gallic acid monohydrate (**11**), thearubigin (**12**), catechin (**13**), epicatechin gallate (**14**), myricitrin (**15**), rutin (**16**), theaflavin (**17**), pyrocatechol (**18**), pyrogallol (**19**), protocatechuic acid (**20**), caffeic acid (**21**), resveratrol (**22**).

EGCg (**2**) (256±141), castalagin (**5**) (333±157), epicatechin gallate (ECg; **14**) (371±252), theaflavin (**17**) (287±188), catechol (**18**) (230±130), and pyrogallol (**19**) (94±91) had comparatively strong activity. Procyanidins (**8**) (1301±797), theaflavins (**9**) (976±522), loquat-treated green tea polyphenols (**10**) (1087±642), thearubigin (**12**) (986±609), (+)-catechin (**13**) (1138±947), rutin (**16**) (1325±641), protocatechuic acid (**20**) (1443±840), caffeic acid (**21**) (1475±706), and resveratrol (**22**) (1748±768) had relatively weak potency. Punicalagin (**3**) (601±668), tannic acid (**4**) (426±357), prodelphinidin (**6**) (509±233), geraniin (**7**) (502±349), gallic acid (**11**) (510±394), and myricitrin (**15**) (442±220) showed moderate activity.

Antibacterial Spectrum of Plant Eextracts The susceptibility to plant extracts (mean MIC±S.D., μg/ml) tended to resemble that of purified polyphenols, as outlined above (Table 2). Although group II was relatively susceptible (390±429), group IV (1001±499) showed moderate susceptibility, and group I (1152±501) and group III (1227±494) showed even less susceptibility. There were significant differences in mean MICs between group II and the other groups, and between the mean MICs of group III and group IV ($p<$ 0.01).

When the antibacterial activity (mean MIC±S.D., μg/ml) of each plant extract was compared (Table 4), *C. perfringens* (272±406), *A. hydrophila* (312±329) and *V. vulnificus* (385±504) showed comparatively strong susceptibility. Susceptibility of *B. cereus* (1063±629), *B. subtilis* (2196±883), *L. monocytogenes* (1928±826), *C. freundii* (1156±542), *E. coli* (1926±848), *P. mirabilis* (1210±607), *P. vulgaris* (992±531), *S.* Anatum (1733±770), *S. arizonae* (1415±558), *S. flexneri* (1436±743), *S. sonnei* (1282±615), *Y. enterocolitica* (1141±581), *P. aeruginosa* (1144±783), *P. cichorii* (995±425), *P. marginalis* (1351±812), *P. viridiflava* (862±682) and *X. campestris* (974±550) was relatively weak. *S. aureus* (664±554), *C. michiganensis* (790±563), *V. parahaemolyticus* (474±490), *K. pneumoniae* (512±291), *E. carotovora* (690±462), and *A. tumefaciens* (679±322) showed moderate susceptibility.

Antibacterial Activity of Plant Extracts The antibacterial activity (mean MIC±S.D., μg/ml) was compared for 26 plant extracts (Table 4). Extracts derived from fresh green tea leaves (**24**) (492±347), *Sapium sebiferum* (**27**) (746±492), *Camellia japonica* (**28**) (783±485), *Stachyurus praecox* (**29**) (636±514), *Castanea crenata* (**31**) (637±296), *Myrica rubra* (**36**) (744±389) and *Paeonia lactiflora* (**37**) (690±764) showed moderate but effective activity. These plant extracts belonged to the catechin, ellagitannin or gallotannin groups, except for **36**, which was classified in the flavonoid group. Of the other 19 species of plant, extracts derived from fermented green tea leaves (**23**), *Bischofia javanica* (**25**), *Elaeocarpus sylvestris* var. *ellipticus* (**26**), *Cornus brachypoda* (**30**), *Fragaria grandiflora* (**32**), *Citrus unshiu* (**33**), *Liquidambar formosana* (**34**), *Fragaria grandiflora* (**35**), *Distylium racemosum* (**38**), *Castanopsis cuspidata* (**39**), *Cryptomeria japonica* (**40**), *Pinus pinaster* (**41**), *Chamaecyparis obtuse* (**42**), *Acacia dealbata* (**43**), *Cinnamonum camphora* (**44**), *Diospyros kaki* (**45**), *Eriobotrya japonica* (**46**), *Pasania edulis* (**47**) and *Vitis vinifera* (**48**) showed a mean MIC >800 μg/ml (Table 4).

DISCUSSION

Antibacterial Spectrum of Polyphenols Many reports on the antibacterial activity of polyphenols have been published[4,9—12]; however, the relationship between polyphenol structure and antibacterial activity has not been clearly demonstrated because these activities were measured by different methods or evaluated by different criteria. In addition, some researchers have found that tea polyphenol activity is stronger against Gram-positive bacteria,[5,13,14] although this is not fully accepted.[4] We aimed to clarify this problem using various species of bacteria and polyphenols with different structures, using the MIC method. In purified polyphenols, the group I Gram-positive bacteria showed comparatively low susceptibility, and there was no significant difference between the group I and group III Enterobacteriaceae. Whether to purified polyphenols or plant extracts, group III had the lowest sensitivity among the four bacterial groups used (Table 2, Fig. 2). In our previous study,[12] Gram-positive *S. aureus* showed high susceptibility to 10 different polyphenols. In the present study, it showed moderate susceptibility to 48 different purified polyphenols or plant extracts. Among other Gram-positive bacteria, the susceptibility of *C. perfringens* was comparatively high, that of *C. michiganensis* was moderate, and *B. cereus*, *B. subtilis* and *L. monocytogenes* showed only low susceptibility. The group II, which includes not only the genus *Vibrio* but also the genus *Aeromonas*, generally showed high susceptibility to both pure polyphenols and extracts (Fig. 2), and there was a significance difference in the mean MIC between this group and the other three groups (Table 2). This result was consistent with our previous study[12] and the other publications.[4,5] The group III Enterobacteriaceae showed the lowest sensitivity, compared to other groups, to both pure polyphenols and extracts, and the mean MIC of each bacterial species in this group also showed a similar tendency (Table 2, Fig. 2). Group IV, which includes the Gram-negative aerobic bacteria, generally showed weak or moderate activity (Fig. 2). Based on these results, we conclude that Gram-staining does not correlate with antimicrobial potency, and polyphenol susceptibility of bacteria growing in Mueller–Hinton medium depends on the bacterial species. In addition, it is likely that in the Gram-negative bacteria, at least the Vibrionaceae show significantly high susceptibility to polyphenols. On the other hand, the Enterobacteriaceae are characterized as a low-susceptibility group, and plant disease-causing bacteria are a moderate-susceptibility group.

Relationship between Antibacterial Activity and The Trihydroxyphenyl Group of Polyphenols In our previous study,[12] it was deduced that the presence of 3,4,5-trihydroxyphenyl groups (the pyrogallol group) is related to antibacterial activity, when comparing the mean MIC of 10 purified polyphenols. In the previous study, there were a limited number of polyphenols, therefore, the structure–activity relationship could not be clearly shown. In the present study, we compared the antibacterial activity of polyphenols more systematically, using various compounds containing pyrogallol, catechol and resorcinol groups.

When the structure of catechin derivatives, EGC (**1**), EGCg (**2**), (+)-catechin (**13**) and ECg (**14**) was compared, the number of catechol groups was zero, zero, one and one,

Table 4. Mean Minimum Inhibitory Concentration (MIC) of Plant Extracts against Bacterial Species

	Bacterial group[a)]	MIC (μg/ml) in plant extracts												
		23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
Group I	*Bacillus cereus*	1600	533	800	1067	1333	933	800	667	800	1067	3200	1600	1067
	Bacillus subtilis	2133	800	3200	1600	2667	133	1600	2667	1600	2667	3200	2667	2667
	Clostridium perfringens	133	50	133	67	133	133	133	167	133	83	2133	133	67
	Listeria monocytogenes	1333	400	2667	933	1067	1867	2667	2133	1067	667	3200	2133	2133
	Staphylococcus aureus	667	400	533	400	400	400	400	400	400	533	3200	800	667
	Clavibacter michiganensis	800	400	533	533	867	1067	667	400	667	400	3200	667	400
	Mean MIC±S.D.	1111±719	431±243	1311±1286	767±546	1078±893	756±673	1044±937	1072±1054	778±516	903±922	3022±435	1333±965	1167±1024
Group II	*Aeromonas hydrophila*	267	267	200	200	200	200	167	200	233	467	1867	233	400
	Vibrio parahaemolyticus	333	267	333	67	200	100	100	267	183	467	2667	133	533
	Vibrio vulnificus	333	133	400	400	267	200	267	267	400	333	2667	400	400
	Mean MIC±S.D.	311±38	222±77	311±102	222±168	222±38	167±58	178±84	244±38	272±113	422±77	2400±462	256±135	444±77
Group III	*Citrobacter freundii*	667	333	800	667	800	800	800	1200	800	800	2667	1067	2133
	Escherichia coli	2133	1600	1067	800	1067	1600	800	2133	667	1867	3200	1600	2133
	Klebsiella pneumoniae	400	200	533	200	400	533	400	400	533	200	1600	600	800
	Proteus mirabilis	1067	533	667	1867	800	800	667	1067	667	800	3200	2667	1333
	Proteus vulgaris	1067	400	667	800	800	800	467	933	533	1067	3200	1333	1067
	Salmonella Anatum	2133	1067	800	1067	800	1600	800	1600	800	1867	3200	2133	2133
	Salmonella arizonae	1067	1067	800	1067	800	1067	800	1333	800	1067	2667	1600	1600
	Shigella flexneri	1067	800	800	1867	800	800	467	933	800	1067	3200	2133	1200
	Shigella sonnei	800	333	800	1067	800	1600	667	1333	800	667	2187	1600	2133
	Yersinia enterocolitica	667	333	800	1067	800	800	800	933	800	667	3200	1600	1333
	Erwinia carotovora	667	267	400	533	400	400	400	533	400	533	2667	800	667
	Mean MIC±S.D.	1067±572	630±447	739±172	1000±507	752±191	982±431	642±175	1127±482	691±144	964±516	2817±532	1558±607	1503±560
Group IV	*Pseudomonas aeruginosa*	1333	533	400	533	667	533	533	1067	667	1067	3200	1067	1333
	Pseudomonas cichorii	667	267	800	933	800	667	533	1133	667	867	2133	1067	1200
	Pseudomonas marginalis	800	800	1467	667	667	1067	467	800	667	800	3200	1067	1067
	Pseudomonas viridiflava	400	400	400	533	400	800	267	667	533	400	3200	667	533
	Agrobacterium tumefaciens	300	200	667	933	533	667	400	333	400	300	1067	1067	400
	Xanthomonas campestris	800	400	667	933	933	800	467	400	533	800	3200	1067	667
	Mean MIC±S.D.	717±366	433±214	733±393	756±201	667±189	756±182	444±100	733±332	578±109	706±294	2667±892	1000±163	867±384
Total	Mean MIC±S.D.	909±578	492±347	821±684	800±481	746±492	783±485	636±514	922±652	637±296	828±572	2782±602	1227±719	1156±706

Table 4. continued

	Bacterial group	MIC (μg/ml) in plant extracts													Total
		36	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	
Group I	*Bacillus cereus*	1067	167	1333	1067	600	400	533	1600	933	1333	2133	667	333	1063±629
	Bacillus subtilis	1067	167	2667	2667	3200	2667	2133	2133	3200	2133	2133	3200	2133	2196±883
	Clostridium perfringens	133	67	200	333	117	133	533	533	533	300	133	267	300	272±406
	Listeria monocytogenes	1067	667	2667	1600	3200	2133	1867	2133	2667	2667	1867	3200	2133	1928±826
	Staphylococcus aureus	333	133	400	667	667	800	533	667	1067	800	667	800	533	664±554
	Clavibacter michiganensis	533	67	800	533	800	933	1067	667	1200	800	1067	1067	400	790±563
	Mean MIC ±S.D.	700±421	211±228	1344±1095	1144±871	1431±1390	1178±1003	1111±724	1289±757	1600±1070	1339±900	1333±839	1533±1317	972±903	
	Aeromonas hydrophila	200	100	200	167	333	333	200	267	333	267	133	267	400	312±329
	Vibrio parahaemolyticus	133	67	100	267	267	933	200	267	400	533	400	267	533	385±504
	Vibrio vulnificus	133	67	267	333	1067	400	400	400	667	667	533	533	400	474±490
	Mean MIC±S.D.	156±38	78±19	189±84	256±84	556±444	556±329	267±115	311±77	467±176	489±204	356±204	356±154	444±77	
Group III	*Citrobacter freundii*	800	1600	800	1067	1067	1867	1333	800	1600	1867	800	1600	1333	1156±542
	Escherichia coli	600	2400	1600	1600	3200	3200	3200	1333	2667	2133	1600	3200	2667	1926±848
	Klebsiella pneumoniae	600	500	533	200	400	1000	400	600	533	400	200	533	600	512±291
	Proteus mirabilis	1067	1333	667	1333	1067	1333	1333	1067	1333	1067	1333	800	1600	1210±607
	Proteus vulgaris	800	800	533	667	800	1333	1333	1067	1333	1333	1067	800	800	992±531
	Salmonella Anatum	1067	2667	1067	1333	1600	2667	2667	800	2667	2133	1067	2667	2667	1733±770
	Salmonella arizonae	800	2400	1067	1333	1600	2133	2133	1333	2133	1067	1067	2133	1867	1415±558
	Shigella flexneri	1867	333	800	533	2133	2667	1867	2133	2133	1333	1867	2133	1600	1436±743
	Shigella sonnei	800	667	800	533	1600	1867	2133	1600	2133	1333	800	2133	2133	1282±615
	Yersinia enterocolitica	1067	533	800	933	1067	1600	2133	1067	1600	1067	1067	1600	1333	1141±581
	Erwinia carotovora	533	533	400	333	800	800	533	1067	1067	933	533	800	933	690±462
	Mean MIC±S.D	909±370	1252±881	824±331	897±471	1394±771	1861±752	1733±850	1170±425	1745±668	1333±530	1036±465	1673±869	1594±697	
Group IV	*Pseudomonas aeruginosa*	533	267	533	1067	1067	2133	3200	1067	1600	1067	533	1600	2133	1144±783
	Pseudomonas cichorii	933	333	800	600	1067	1600	1333	800	1600	1200	1067	1600	1200	995±425
	Pseudomonas marginalis	667	400	1867	800	2400	1867	1067	1067	2400	1600	1867	2400	3200	1351±812
	Pseudomonas viridiflava	667	267	1067	667	1333	933	800	1333	1200	800	667	2667	800	862±682
	Agrobacterium tumefaciens	933	533	667	267	800	1067	1067	1067	1067	400	1067	1067	400	679±322
	Xanthomonas campestris	933	867	1067	800	800	1333	800	1067	1867	800	933	1067	1333	974±550
	Mean MIC±S.D.	778±177	444±230	1000±475	700±266	1244±600	1489±465	1378±915	1067±169	1622±480	978±410	1022±467	1733±669	1511±1011	
Total	Mean MIC±S.D.	744±389	690±764	912±665	835±569	1271±887	1467±817	1338±884	1074±527	1536±784	1155±628	1023±592	1503±961	1299±851	

a) Each bacterial group is the same as in Table 2. Fermented tea leaves (**23**), fresh tea leaves (**24**), *Bischofia javanica* (**25**), *Elaeocarpus sylvestris* var. *ellipticus* (**26**), *Sapium sebiferum* (**27**), *Camellia japonica* (**28**), *Stachyurus praecox* (**29**), *Cornus brachypoda* (**30**), *Castanea crenata* (**31**), *Fragaria grandiflora* (**32**), *Citrus unshiu* (**33**), *Liquidambar formosana* (**34**), *Fragaria grandiflora* (**35**), *Myrica rubra* (**36**), *Paeonia lactiflora* (**37**), *Distylium racemosum* (**38**), *Castanopsis cuspidata* (**39**), *Cryptomeria japonica* (**40**), *Pinus pinaster* (**41**), *Chamaecyparis obtuse* (**42**), *Acacia dealbata* (**43**), *Cinnamonum camphora* (**44**), *Diospyros kaki* (**45**), *Eriobotrya japonica* (**46**), *Pasania edulis* (**47**), *Vitis vinifera* (**48**).

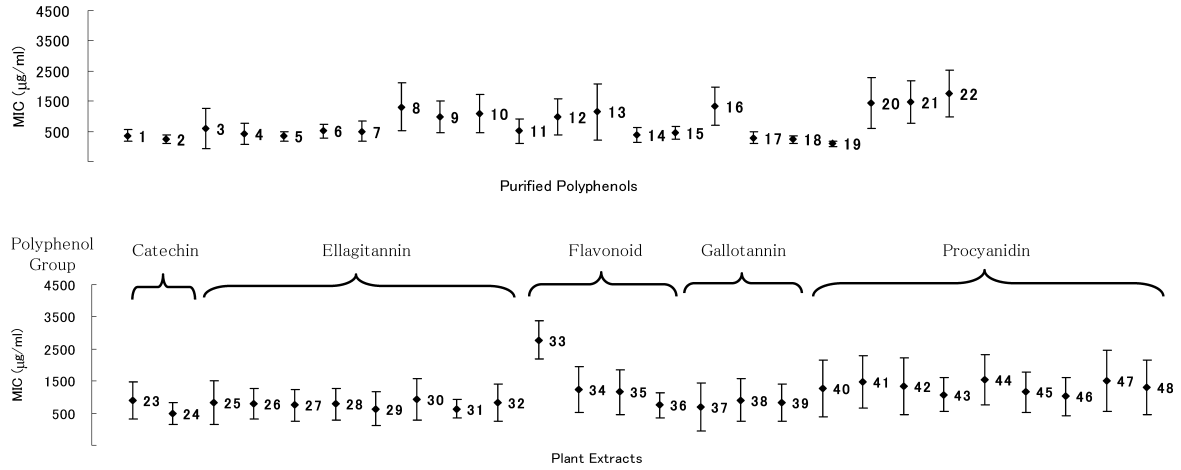

Fig. 3. Mean Minimum Inhibitory Concentration (MIC) of Purified Polyphenols and Plant Extracts

Epigallocatechin (**1**), epigallocatechin-3-*O*-gallate (**2**), punicalagin (**3**), tannic acid (**4**), castalagin (**5**), prodelphinidins (**6**), geraniin (**7**), procyanidins (**8**), theaflavins (**9**), loquat-treated green tea polyphenols (**10**), gallic acid (**11**), thearubigin (**12**), catechin (**13**), epicatechin gallate (**14**), myricitrin (**15**), rutin (**16**), theaflavin (**17**), pyrocatechol (**18**), pyrogallol (**19**), protocatechuic acid (**20**), caffeic acid (**21**), resveratrol (**22**), fermented tea leaves (**23**), fresh tea leaves (**24**), *Bischofia javanica* (**25**), *Elaeocarpus sylvestris* var. *ellipticus* (**26**), *Sapium sebiferum* (**27**), *Camellia japonica* (**28**), *Stachyurus praecox* (**29**), *Cornus brachypoda* (**30**), *Castanea crenata* (**31**), *Fragaria grandiflora* (**32**), *Citrus unshiu* (**33**), *Liquidambar formosana* (**34**), *Fragaria grandiflora* (**35**), *Myrica rubra* (**36**), *Paeonia lactiflora* (**37**), *Distylium racemosum* (**38**), *Castanopsis cuspidata* (**39**), *Cryptomeria japonica* (**40**), *Pinus pinaster* (**41**), *Chamaecyparis obtuse* (**42**), *Acacia dealbata* (**43**), *Cinnamonum camphora* (**44**), *Diospyros kaki* (**45**), *Eriobotrya japonica* (**46**), *Pasania edulis* (**47**), *Vitis vinifera* (**48**). Vertical bars represent the mean±S.D.

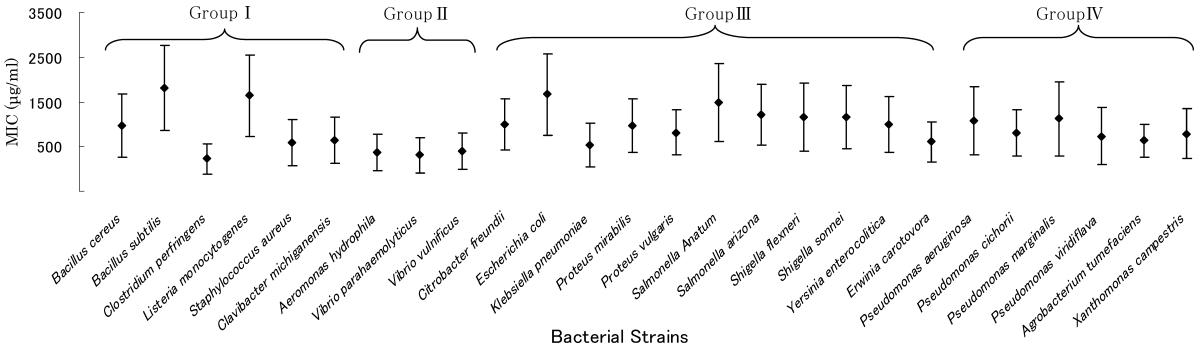

Fig. 2. Mean Minimum Inhibitory Concentration (MIC) for Each Bacterial Strain

Vertical bars represent the mean±S.D.

the number of resorcinol groups was one in all compounds, and the number of pyrogallol groups was one, two, zero and one, respectively. Since the order of antibacterial activity was **2**>**1**≒**14**>**13**, the activity was well-correlated with the number of pyrogallol rings (Table 3). Prodelphinidins (**6**) have similar structures to procyanidins (**8**) except for their hydroxyphenyl group (Fig. 1). **6**, which have the pyrogallol groups, showed stronger activity than **8** with the catechol groups. Some similarities were observed between gallic acid (**11**) and protocatechuic acid (**20**), between myricitrin (**15**) and rutin (**16**), and between pyrogallol (**19**) and catechol (**18**) (Fig. 1). The polyphenols with the pyrogallol groups all showed stronger activity than those with the catechol group (Fig. 3). In addition, protocatechuic acid (**20**), caffeic acid (**21**) and resveratrol (**22**) which have only the resorcinol or catechol group, showed weak activity (Figs. 1, 3). These results reveal that the pyrogallol group is important for the antibacterial activity of the polyphenols, and the catechol and resorcinol groups are less important.

Comparison of The Antibacterial Activity of Plant Extracts The major constituents of some plant extracts, which were classified into the catechin group, the ellagitannin group and the gallotannin group, all have pyrogallol groups and these extracts showed comparatively strong antibacterial activity in this study (Table 4, Fig. 3). For example, extracts of fresh tea leaves (**24**), containing mainly EGC (**1**) and EGCg (**2**); *Stachyurus praecox* (**29**), containing mainly ellagitannins; *Castanea crenata* (**31**), containing ellagitannins and gallotannins; and *Paeonia lactiflora* (**37**), containing gallotannins showed moderate activity and each main constituent all has pyrogallol groups. And this confirms the importance of the pyrogallol group. On the other hand, the main constituents of *Cryptomeria japonica* (**40**), *Pinus pinaster* (**41**), *Chamaecyparis obtuse* (**42**), *Acacia dealbata* (**43**), *Cinnamonum camphora* (**44**), *Diospyros kaki* (**45**), *Eriobotrya japonica* (**46**), *Pasania edulis* (**47**) and *Vitis vinifera* (**48**) were the procyanidins which contain catechol and resorcinol rings. The activity of these plant extracts was generally weak (Fig. 3), as expected. *Citrus unshiu* (**33**) contains flavonoids, and *Liquidambar formosana* (**34**) and *Fragaria grandiflora* (**35**) contain procyanidins or flavonoids with the catechol and resorcinol groups as major constituents, although these plants also contain hydrolyzable tannins. The comparatively strong activity of *Myrica rubra* (**36**) seems to be due to the activity of myricitrin (**15**) and prodelphinidins (**6**) with the pyrogallol groups. These results show that the high antibac-

terial potential of plant extracts can be predicted, according to whether the polyphenols have pyrogallol groups present.

Relationship between Antibacterial Activity and Other Polyphenol Functions There are several hypotheses on the antibacterial activity of polyphenols.[1,2,13—15] For example, Ikigai *et al.*[13] suggested that polyphenols adsorb on to the surface of the bacterial cell wall and act to inhibit or kill the bacteria physically. Arakawa *et al.*[14] suggested that oxidative polyphenols generate hydrogen peroxide which may mediate antibacterial activity. As yet, there is no clear consensus concerning these mechanisms.

In the present study, the small mean MIC values (94—601 μg/ml) of the purified polyphenols which contain pyrogallol groups (Table 3) indicated the importance of this type of aromatic ring in strong antibacterial activity. Exceptionally, theaflavin (**17**) and catechol (**18**), which do not have pyrogallol rings, also showed strong antibacterial activity. The unique benzotropolone ring of **17** is related to hinokitiol, an essential oil having strong antibacterial activity; therefore, the structural similarity may account for the activity of **17**. Akagawa *et al.*[17] reported that catechol generates hydrogen peroxide at pH over 7.4 and pyrogallol also generates larger content of H_2O_2 at pH over 6.0 than that of catechol. This is supported to evaluate H_2O_2 generation from oxidative epigallocatechin gallate by Arakawa *et al.*[14] In this study H_2O_2 might also be generated accompanied by oxidation of catechol in the culture medium (pH 6.8) to show high antibacterial activity. On the other hand, it is clear that nine different purified polyphenols with catechol or resorcinol groups do not show antibacterial activity.

Our results strongly suggested that plant polyphenols with pyrogallol groups show higher antibacterial activity compared to those with catechol or resorcinol groups; however, there was no clear relationship between the number of pyrogallol groups and the antibacterial activity of the polyphenols. When the antibacterial activity of compounds or plant extracts cannot simply be estimated by the number of hydroxyphenyl groups, it is necessary to take into account other factors, such as medium pH and bacterial properties.

Acknowledgments The authors thank Dr. Hisatoshi Kaku (National Institute of Agrobiological Sciences) for donating the strains used for this study.

REFERENCES

1) Scalbert A., *Phytochemistry*, **30**, 3875—3883 (1991).
2) Hamilton M. J. M. T., *Antimicrob. Agents Chemother.*, **39**, 2375—2377 (1995).
3) Ryu E., *Int. J. Zoon.*, **7**, 164—170 (1980).
4) Hara Y., Ishigami T., *Nippon Shokuhin Kogyo Gakkaishi*, **36**, 996—999 (1989).
5) Toda M., Okubo S., Hiyoshi R., Shimamura T., *Lett. Appl. Microbiol.*, **8**, 123—125 (1989).
6) Kudo Y., Okubo T., Tanaka S., Chu D.-C., Juneja L. R., Saito N., Konishi Y., *Biocontrol Sci.*, **6**, 57—61 (2001).
7) Kudo Y., Kobayashi A., Konishi Y., Kondo K., *J. Food Protection*, **67**, 2820—2824 (2004).
8) Hsieh P.-C., Mau J.-L., Huang S.-H., *Food Microbiology*, **18**, 35—43 (2001).
9) Toda M., Okubo S., Ikigai H., Shimamura T., *Jpn. J. Bacteriol.*, **45**, 561—566 (1990).
10) Hattori M., Kusumoto I.T., Namba T., Ishigami T., Hara Y., *Chem. Pharm. Bull.*, **38**, 717—720 (1990).
11) Fukai K., Ishigami T., Hara Y., *Agric. Biol. Chem.*, **55**, 1895—1897 (1991).
12) Taguri T., Tanaka T., Kouno I., *Biol. Pharm. Bull.*, **27**, 1965—1969 (2004).
13) Ikigai H., Nakae T., Hara Y., Shimamura T., *Biochim. Biophys. Acta*, **1147**, 132—136 (1993).
14) Arakawa H., Maeda M., Okubo S., Shimamura T., *Biol. Pharm. Bull.*, **27**, 277—281 (2004).
15) Ikigai H., Hara Y., Otsuru H., Shimamura T., *Jpn. J. Chemother.*, **46**, 179—183 (1998).
16) Society of Industrial-Technology for Antimicrobial Articles: ⟨http://kohkin.net/m9/siaa/recent/ck.cgi?command=pub/index2::main&pg=download, PSJ Web, 27 December 2005.
17) Akagawa M., Shigematsu T., Suyama K., *Biosci. Biotechnol. Biochem.*, **67**, 2632—2640 (2003).