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# Anti-*Salmonella* Activity of Alkyl Gallates†

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A series of alkyl gallates (3,4,5-trihydroxybenzoates) was synthesized and tested for their antibacterial activity against *Salmonella choleraesuis*. Nonyl ($C_9$) and octyl ($C_8$) gallates were noted to be the most effective against this food-borne bacterium, each with a minimum bactericidal concentration (MBC) of 12.5 $\mu$g/mL, followed by decyl ($C_{10}$) gallate, with a MBC of 25 $\mu$g/mL. Dodecyl ($C_{12}$) gallate exhibited activity against *S. choleraesuis*, with a MBC of 50 $\mu$g/mL. Propyl ($C_3$) gallate showed no activity against *S. choleraesuis* up to 3200 $\mu$g/mL. The length of the alkyl group is not a major contributor but plays a role in eliciting the activity to a large extent. The same series of alkyl gallates, regardless of alkyl chain length, all showed nearly the same potent scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl radical, indicating that the length of the alkyl group is not associated with the activity.

**KEYWORDS: Antibacterial activity; *Salmonella choleraesuis*; *Proteus vulgaris*; alkyl gallates; respiratory inhibition; antioxidant activity**

## INTRODUCTION

Due to changes in the marketing of food products, the use of chemical additives in foods, such as antioxidant and antimicrobial agents, has become increasingly important. For example, today foods produced in one area are often shipped to another area for processing and to several other areas for distribution. Several months or years may elapse from the time the food is processed until it is consumed. New methods of food preparation have also increased the need for these additives. This prompted us to design a chemical that satisfies both needs. Two chemicals, antioxidative gallic acid and antimicrobial aliphatic alcohols (alkanols), were selected for this study. It should be noted that alkanols do not have any antioxidant activity, while gallic acid does not exhibit any antimicrobial activity. However, the esters of gallic acid and these alcohols are expected to exhibit both activities. The selection of alkyl gallates is based on safety as the first priority. The "hydrolyzable" ester group can prevent undesirable side effects, particularly the endocrine-disrupting activity of environmentally persistent estrogen mimics (*1*) such as alkylphenolic compounds (*2*).

The salmonellae are Gram-negative non-spore-forming rods. There are over 2500 serovars of *Salmonella*, all of which are pathogenic to humans. Salmonellosis may result following the ingestion of viable cells of a member of the genus *Salmonella*. It is the most frequently occurring bacterial food infection and is a commonly occurring bacterial food-borne illness. Due to the adaptability of Gram-negative bacteria, they can easily develop resistance to commonly used antibiotics (*3*, *4*). This resistance involves enzymatic inactivation in resistant bacteria (*5*) and is often transferred to other bacteria by a variety of gene-transfer mechanisms. Because of drug resistance, there is a great need for effective antibacterial agents against Gram-negative bacteria with new modes of action. The ideal antimicrobial agent was recently suggested to be produced by rational design (*6*). In a previous report, the primary fungicidal activity of alkyl gallates against *Saccharomyces cerevisiae* was described as resulting from its ability to act as a nonionic surface-active agent (surfactant) (*7*). Antibacterial agents which act primarily as surfactants have the potential to fill this need, since they target the extracytoplasmic region and thus do not need to enter the cell, thereby avoiding most cellular pump-based resistance mechanisms. In the current experiment, *Salmonella choleraesuis* was selected since this bacterium is one of the most frequent causes of septicemia, even though septicemia can be caused by any *Salmonella* bacterium (*8*).

## MATERIALS AND METHODS

**Chemicals.** Alcohols were available from our previous work (*9*). Gallic acid, propyl gallate, octyl gallate, dodecyl gallate, and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Gentamicin and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co. (St. Louis, MO). For the experiment, all the compounds were first dissolved in *N*,*N*-dimethylformamide (DMF) that was purchased from EM Science (Gibbstown, NJ).

**Synthesis.** To a solution of gallic acid (2.00 mM) and alcohol (2.00 mM) in tetrahydrofuran (THF) (6 mL), cooled at 0 °C, was added a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC) (4.2 mM) in THF (6 mL). After the solution was stirred for 20 h, the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate several times and filtered. The filtrate was washed successively with

† In honor of Professor Andrew S. Kende's 70th birthday.

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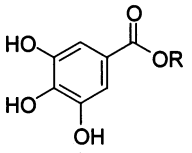
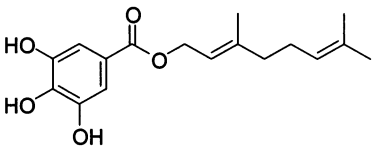


**1**: R = $CH_2(CH_2)_6CH_3$
**2**: R = $CH_2(CH_2)_{10}CH_3$
**3**: R = $CH_2(CH_2)_{11}CH_3$
**4**: R = $CH_2(CH_2)_9CH_3$
**5**: R = $CH_2(CH_2)_8CH_3$
**6**: R = $CH_2(CH_2)_7CH_3$
**7**: R = $CH_2(CH_2)_5CH_3$
**8**: R = $CH_2(CH_2)_4CH_3$



**9**

**Figure 1.** Chemical structures of alkyl gallates and related compounds.

dilute aqueous citric acid solution, saturated aqueous $NaHCO_3$ solution, and water, dried over $MgSO_4$, and evaporated. The crude products were purified by chromatography ($SiO_2$; elution with $CHCl_3$−MeOH, 98:2, v/v). Structures of the synthesized esters were established by spectroscopic methods (IR, MS, and NMR) (**Figure 1**). Their analogues, described in this paper, were synthesized in the same manner.

*Tridecyl gallate (tridecyl 3,4,5-trihydroxybenzoate)* (**3**) was obtained in 77% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.16 (s, 2H, ArH); IR (KBr) 3520, 3495, 3010, 2960, 1686, 1615, 1480, 1420, 1395, 1270, 1140 cm$^{-1}$.

*Dodecyl gallate (dodecyl 3,4,5-trihydroxybenzoate)* (**2**) was obtained in 65% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.16 (s, 2H, ArH); IR (KBr) 3515, 3490, 3000, 2960, 1680, 1610, 1480, 1395, 1270, 1145 cm$^{-1}$. Dodecyl gallate was also purchased from Sigma Chemical Co. but was recrystallized prior to use.

*Undecyl gallate (undecyl 3,4,5-trihydroxybenzoate)* (**4**) was obtained in 63% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.16 (s, 2H, ArH); IR (KBr) 3500, 3450, 2960, 2890, 1685, 1620, 1480, 1395, 1280, 1160 cm$^{-1}$.

*Decyl gallate (decyl 3,4,5-trihydroxybenzoate)* (**5**) was obtained in 74% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.16 (s, 2H, ArH); IR (KBr) 3391, 2924, 1686, 1615, 1448, 1312, 1258, 1028 cm$^{-1}$; HRMS-EI ($m/z$) [M]$^+$ calcd for $C_{17}H_{26}O_5$ 310.1778, found 310.1788.

*Nonyl gallate (nonyl 3,4,5-trihydroxybenzoate)* (**6**) was obtained in 86% yield as colorless needles from benzene−*n*-pentane: mp 96.7−97.3 °C; $^1$H NMR (300 MHz, $CDCl_3$−$CD_3OD$) $\delta$ 0.88 (t, $J$ = 6.6 Hz, 3H, $-CH_3$), 4.25 (t, $J$ = 6.6 Hz, 2H, $-OCH_2$), 7.21 (s, 2H, ArH); IR (KBr) 3489, 3439, 3333, 1670, 1625, 1604, 1531, 1300, 1256, 1024 cm$^{-1}$; HRMS-EI ($m/z$) [M]$^+$ calcd for $C_{16}H_{24}O_5$ 296.1630, found 296.1624.

*Octyl gallate (octyl 3,4,5-trihydroxybenzoate)* (**1**) was obtained in 89% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.15 (s, 2H, ArH); IR (KBr) 3325, 2926, 1686, 1614, 1468, 1314, 1246, 1028 cm$^{-1}$; HRMS-EI ($m/z$) [M]$^+$ calcd for $C_{15}H_{22}O_5$ 282.1469, found 282.1473.

*Heptyl gallate (heptyl 3,4,5-trihydroxybenzoate)* (**7**) was obtained in 71% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.24 (t, $J$ = 6.5 Hz, 2H, $-OCH_2$), 7.16 (s, 2H, ArH); IR (KBr) 3389, 2920, 1685, 1620, 1448, 1320, 1258, 1140 cm$^{-1}$.

*Hexyl gallate (hexyl 3,4,5-trihydroxybenzoate)* (**8**) was obtained in 58% yield as a colorless solid: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.88 (t, $J$ = 6.5 Hz, 3H, $-CH_3$), 4.18−4.28 (br, 2H, $-OCH_2$), 6.4−5.8 (br, 3H, exchangeable with $D_2O$, −ArOH), 7.20 (s, 2H, ArH); IR (KBr) 3389, 2932, 1688, 1615, 1449, 1314, 1258, 1026 cm$^{-1}$.

*Geranyl gallate (3,7-dimethylocta-2,6-dienyl 3,4,5-trihydroxybenzoate)* (**9**) was obtained in 57% yield as colorless powder: mp 67.0−68.5 °C; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 1.60 (s, 3H, $-CH_3$), 1.67 (d, $J$ = 0.6 Hz, 3H, $-CH_3$), 1.75 (d, $J$ = 1.2 Hz, 3H, $-CH_3$), 2.08 (m, 4H, $-CH_2$), 4.79 (d, $J$ = 7.2 Hz, 2H, $-OCH_2$), 5.09 (m, 1H, −CH), 5.43 (qt, $J$ = 7.2, 1.2 Hz, 1H, −CH), 6.12 (br s, 3H, −OH), 7.31 (s, 2H, ArH); IR (KBr) 3549, 3406, 3288, 1686, 1614, 1537, 1312, 1244, 1229, 1198, 1022 cm$^{-1}$; EI-MS ($m/z$) 306 (M$^+$), 237, 170, 153 (base peak), 136, 121, 93, 69, 41; HRMS−FAB ($m/z$) [M + H]$^+$ calcd for $C_{17}H_{23}O_5$ 307.1546, found 307.1561.

**Test Strains.** The test strains, *Salmonella choleraesuis* ATCC 35640, *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Enterobacter aerogenes* ATCC 13048, and *Proteus vulgaris* ATCC 13315, used for this study were purchased from American Type Culture Collection (Manassas, VA). The strain of *Ps. aeruginosa* IFO 3080 was available from our previous work.

**Medium.** NYG broth (0.8% nutrient broth, 0.5% yeast extract, 0.1% glucose) was used for the antibacterial assay. Nutrient broth was obtained from BBL Microbiology System (Cockeysville, MD). Yeast extract was purchased from Difco Lab (Detroit, MI).

**Precultivation.** The cells of *S. choleraesuis* ATCC 35640 were precultured in 3 mL of NYG broth without shaking at 37 °C for 16 h. The preculture was used for the following antibacterial assay and time kill study.

**Antibacterial Assay.** Broth macrodilution methods were followed as previously described (*9*) with slight modifications. Briefly, serial 2-fold dilutions of the test compounds were prepared in DMF, and 30 $\mu$L of each dilution was added to 3 mL of NYG broth. These were inoculated with 30 $\mu$L of preculture of *S. choleraesuis*. After incubation of the cultures at 37 °C for 24 h, the minimum inhibitory concentration (MIC) was determined as the lowest concentration of the test compound that demonstrated no visible growth. The minimum bactericidal concentration (MBC) was determined as follows. After the determination of the MIC, 100-fold dilutions with drug-free NYG broth from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the test compound that was not visible in the drug-free cultivation. The concentration of DMF in each medium was always 1%. The highest concentration tested was 3200 $\mu$g/mL unless otherwise noted.

**Measurement of Oxygen Uptake.** Exponentially growing *Ps. aeruginosa* IFO 3080 cells were harvested and washed with saline by centrifugation. The cells were suspended in 50 mM phosphate buffer (pH 7.0) to give approximately 1 mg of dry cells per milliliter. The test compound dissolved in DMSO was added to the reaction mixture, and the $O_2$ consumption was measured polarographically at 30 °C with an OBH 100 oxygen electrode (*10*).

**Preparation of Bacterial Cell Membrane.** Exponentially growing *Ps. aeruginosa* IFO 3080 cells were harvested by centrifugation and then washed twice with distilled water. The cell paste was suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose and 20 mM $MgCl_2$, and then it was disrupted by ultrasound using a Sonifier 450 at 10 kc for 2 min at 4 °C. After centrifugation of the cell suspension at 15000*g* for 20 min, the supernatant was centrifuged at 105000*g* for 90 min. The resultant precipitate was washed by centrifugation at 105000*g* for 60 min with 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose and 10 mM $MgCl_2$. The precipitate was resuspended in the same buffer (*11*).

**Enzyme Assay.** NADH oxidase activity was assayed by measuring the decrease in the absorbance at 340 nm. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.5), 200 $\mu$M NADH, and membrane fraction (equivalent to 2 mg of protein) (*12*).

## RESULTS AND DISCUSSION

In our previous report, propyl ($C_3$), octyl ($C_8$), and dodecyl ($C_{12}$) gallates were tested for their antimicrobial activity against

**Table 1.** Antimicrobial Activity of Propyl ($C_3$), Octyl ($C_8$), and Dodecyl ($C_{12}$) Gallates

| microorganisms tested | MIC and MBC or MFC ($\mu$g/mL)[a] | | |
|---|---|---|---|
| | $C_3$ | $C_8$ | $C_{12}$ |
| *Bacillus subtilis* | 800 (*1600*) | 12.5 (*25*) | 12.5 (*25*) |
| *Brevibacterium ammoniagenes* | 1600 (*3200*) | 25 (*50*) | 12.5 (*25*) |
| *Micrococcus luteus* | 1600 (*3200*) | 12.5 (*25*) | 12.5 (*25*) |
| *Streptococcus mutans* | 400 (*800*) | 50 (*50*) | 100 (*100*) |
| *Staphylococcus aureus* | 1600 (*3200*) | 25 (*50*) | 12.5 (*25*) |
| *Staph. aureus* (MRSA) | 1600 (*3200*) | 25 (*50*) | 12.5 (*25*) |
| *Propionibacterium acnes* | 800 (*800*) | 25 (*25*) | 6.25 (*6.25*) |
| *Escherichia coli* | 1600 (*1600*) | >800 (>*800*) | >800 (>*800*) |
| *Pseudomonas aeruginosa* | 3200 (>*3200*) | >800 (>*800*) | >800 (>*800*) |
| *Enterobacter aerogenes* | 3200 (>*3200*) | >800 (>*800*) | >800 (>*800*) |
| *Proteus vulgaris* | 400 (*400*) | 25 (*50*) | >800 (>*800*) |
| *Salmonella choleraesuis* | 1600 (>*3200*) | 12.5 (*12.5*) | 25 (*50*) |
| *Saccharomyces cerevisiae* | 3200 (>*3200*) | 25 (*25*) | >1600 (>*1600*) |
| *Zygosaccharomyces bailii* | >3200 (>*3200*) | 50 (*50*) | >1600 (>*1600*) |
| *Candida albicans* | 3200 (>*3200*) | 25 (*25*) | >400 (>*400*) |
| *Aspergillus niger* | >3200 (>*3200*) | 50 (*100*) | >400 (>*400*) |

[a] Numbers in *Italic* type in parentheses are MBC or MFC (minimum fungicidal concentration).

the 16 selected microorganisms. As listed in **Table 1**, octyl gallate was found to exhibit a broad antimicrobial spectrum. It was the only compound active against the fungi tested (*7*). In contrast to octyl gallate, neither propyl nor dodecyl gallates showed any notable fungicidal activity. Octyl gallate was noted to exhibit bactericidal activity against two Gram-negative bacteria, *S. choleraesuis* and *P. vulgaris*, with MBCs of 12.5 (44 $\mu$M) and 50 $\mu$g/mL (177 $\mu$M), respectively. The potency of octyl gallate against *S. choleraesuis* was comparable with that of gentamicin. The differences in the MICs and MBCs of octyl gallate against *S. choleraesuis* and *P. vulgaris* were not more than 2-fold, suggesting that its activity is bactericidal. Dodecyl gallate exhibits activity against *S. choleraesuis* with a MBC of 50 $\mu$g/mL (148 $\mu$M), but not against *P. vulgaris* up to 800 $\mu$g/mL. Propyl gallate also exhibits activity against *P. vulgaris* with a MBC of 400 $\mu$g/mL and against *E. coli* with a MBC of 1600 $\mu$g/mL. Among the three gallates tested, propyl gallate is the only gallate active against *E. coli*, but its weak MBC may prevent practical application. Both *S. choleraesuis* and *P. vulgaris* seem to have different susceptibilities to alkyl gallates possessing different chain lengths. *Ps. aeruginosa*, *Ent. aerogenes*, and *E. coli* are basically tolerant of alkyl gallates, although propyl gallate showed weak bactericidal activity against *E. coli*.

The results obtained indicate that the susceptibility of *S. choleraesuis* and *P. vulgaris* to alkyl gallates is similar to that of Gram-positive bacteria. It is clear that the length of the alkyl group plays a role in eliciting the activity. The hydrophobicity is known to be related to biological action (*14*), but the precise role is still poorly understood. In our continuing efforts to clarify this, alkyl gallates are a superior model for structure and anti-*Salmonella* activity relationship (SAR) study because these molecules possess the same hydrophilic portion, the pyrogallol group, thus distinguishing the role of the hydrophobic alkyl portion. In addition, a series of alkyl gallates and related analogues are readily available. The current study was centered on *S. choleraesuis* ATCC 35640 as a target organism.

A series of alkyl gallates ($C_3$−$C_{13}$) was synthesized and tested for their antibacterial activity against *S. choleraesuis* for comparison. The results are listed in **Table 2**. This food-borne bacterium showed different susceptibility to alkyl gallates possessing different chain lengths. The range of the antibacterial activity of alkyl gallates against *S. choleraesuis* is between 12.5 and 100 $\mu$g/mL, and the MICs and MBCs are nearly the same. Both the MIC and the MBC of the most potent nonyl ($C_9$) gallate are 12.5 $\mu$g/mL (42 $\mu$M), indicating that its activity is bactericidal. Notably, this MBC value is comparable with that of gentamicin. The potency of the bactericidal activity against this food-borne bacterium was increased with each additional $CH_2$ group, up to nonyl gallate. However, the activity did not disappear after the chain length reached the maximum activity. As mentioned previously, dodecyl (lauryl) gallate shows bactericidal activity, with a MBC of 50 $\mu$g/mL, while tridecanyl ($C_{13}$) gallate showed no activity up to 1600 $\mu$g/mL. Thus, the cutoff was made between dodecyl and tridecyl gallates.

**Table 2.** Antibacterial Activity of Gallic Acid and Its Esters against *S. choleraesuis*

| gallates tested | MIC ($\mu$g/mL) | MBC ($\mu$g/mL) | gallates tested | MIC ($\mu$g/mL) | MBC ($\mu$g/mL) |
|---|---|---|---|---|---|
| gallic acid | 1600 | 1600 | $C_{10}$ | 12.5 | 25 |
| $C_3$ | 1600 | >3200 | $C_{11}$ | 25 | 50 |
| $C_6$ | 100 | 100 | $C_{12}$ | 25 | 50 |
| $C_7$ | 50 | 50 | $C_{13}$ | >1600 | –[a] |
| $C_8$ | 12.5 | 12.5 | geranyl gallate | 50 | 50 |
| $C_9$ | 6.25 | 12.5 | gentamicin | 12.5 | 12.5 |

[a] –, not tested.

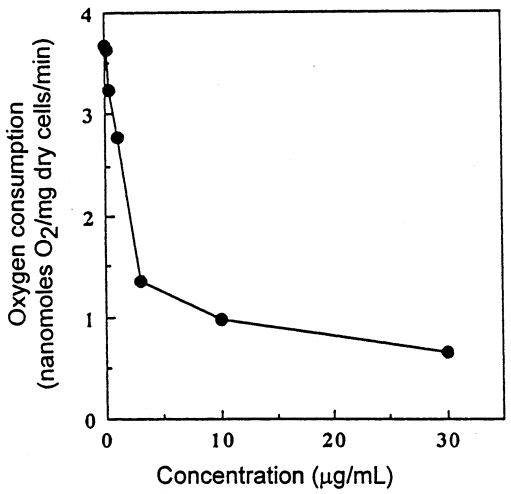



**Figure 2.** Effect of dodecyl gallate on respiratory activity in *Ps. aeruginosa* IFO 3080 cells. Each plot is the mean of triplicate determinations.

Alkyl gallates can be considered as having a head-and-tail structure, similar to alkanols (*9*). Therefore, their mode of antibacterial action was expected to be similar to that of surface-active agents (surfactants). However, the data obtained so far indicate that their antibacterial activity is unlikely to be due mainly to their surfactant property. The activities of the seven alkyl gallates ($C_6$−$C_{12}$) against *S. choleraesuis* were comparable (**Table 2**). More specifically, the antibacterial activity against *S. choleraesuis* did not distinctly increase with every additional $CH_2$ group, indicating that the length of the alkyl group is not largely associated with the potency of the activity. This differs from their antifungal action against *Sac. cerevisiae* (*7*).

During the study to clarify modes of antibacterial action, alkyl gallates were noted to inhibit bacterial respiratory systems. For example, dodecyl gallate was found to inhibit the oxygen consumption of *Ps. aeruginosa* cells when suspensions prepared from the same bacterium cells were incubated with this gallate. The dose−response for respiratory inhibition by dodecyl gallate is shown in **Figure 2**. The same gallate also inhibited *Ps. aeruginosa* NADH oxidase by a membrane fraction prepared

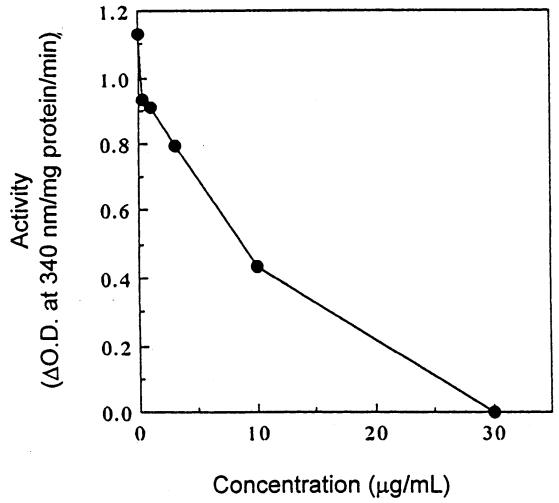



**Figure 3.** Effect of dodecyl gallate on NADH oxidase in a membrane fraction isolated from *Ps. aeruginosa* IFO 3080. Each plot is the mean of triplicate determinations.

from the same bacterial cells, as shown in **Figure 3**. The results observed indicate that dodecyl gallate inhibits the bacterial membrane respiratory chain. It seems that the antibacterial activity of alkyl gallates is due primarily to their ability to inhibit respiration. The assay was performed as previously described (*10*, *15*). It should be noted that dodecyl gallate inhibited the growth of *Ps. aeruginosa* IFO 3080 strain with a MIC of 12.5 μg/mL but not ATCC 33591 strain up to 800 μg/mL. In connection, *Ps. aeruginosa* IFO 3080 strain is not susceptible to most antibiotics of microbial origin, but it is sensitive to some antimicrobial phytochemicals (*15*, *16*). The concentrations of dodecyl gallate found to inhibit bacterial respiratory systems are comparable to those having bactericidal activity against *S. choleraesuis* as well as *Ps. aeruginosa* IFO 3080 strain.

The difference in antibacterial action of alkyl gallates against *S. choleraesuis* needs to be compared with their action against the other Gram-negative bacteria such as *E. coli* and *Ps. aeruginosa*. In the case of bacteria, various enzymes, especially components of energy-converting systems such as electron transport chains (ETCs) and ATPases, are embedded in the membrane lipid bilayers. In the current study, the process by which alkyl gallates reach the active sites in living microorganisms must be taken into account because this is usually neglected in the cell-free experiment. The inner and outer surfaces of the membrane are hydrophilic while the interior is hydrophobic, so the increased lipophilicity of alkyl gallates should affect their movement into the membrane lipid bilayer portions. It seems reasonable to assume that most of the lipophilic dodecyl gallate molecules being dissolved in the medium are incorporated into the lipid bilayers (*17*) without perturbing the lipid (*18*). Once inside the membrane lipid bilayers, alkyl gallates may inhibit the ETC, perhaps by interfering with the redox reactions. The different susceptibilities between *S. choleraesuis* and *Ps. aeruginosa* may be caused by the different permeability of their outer membrane layer, since this plays a major role in the general resistance of Gram-negative bacteria, especially to lipophilic antibiotics. It is known that most Gram-negative bacteria are surrounded by the outer membrane, and this functions as an effective but less specific barrier (*19*). It is logical to assume that most of the lipophilic gallate molecules being dissolved in the medium are incorporated into the outer membrane, and hence cannot reach the ETC in the plasma membrane of *E. coli* and *Ps. aeruginosa*. This may reveal why alkyl gallates are effective against Gram-positive bacteria but not Gram-negative bacteria such as *E. coli* and *Ps. aeruginosa*.

Prokaryotic and eukaryotic microorganisms are known to differ in many ways. For example, the ETC involved in the respiratory chain is located in the cytoplasmic membrane in bacteria, while in fungi it is located in the mitochondria. In the case against fungi, dodecyl gallate can rarely enter into the cytoplasm, and hence cannot reach the mitochondria. This may explain why dodecyl gallate did not show any effect on eukaryotic microorganisms such as *Sac. cerevisiae*, in which respiration depends on a mitochondrial ETC. It appears that microorganisms having different membrane structures showed different susceptibilities to alkyl gallates having different chain lengths. The results obtained may provide a more rational and scientific approach to the design of selective and effective antimicrobial agents.

Among the Gram-negative bacteria tested, *S. choleraesuis* and *P. vulgaris* were rare bacteria susceptible to alkyl gallates. In other words, alkyl gallates fall short of the broad spectrum of activity as far as Gram-negative bacteria are concerned, but they are active against *S. choleraesuis* and *P. vulgaris*. It appears that *S. choleraesuis* and *P. vulgaris* differ from the other Gram-negative bacteria tested in some aspects. If the selective elimination of *S. choleraesuis* and *P. vulgaris* is desirable in food, some alkyl gallates may be considered to be superior. Dodecyl gallate is even more specific against *S. choleraesuis*. In connection with food, one of the most commonly occurring types of food poisoning is caused by the ingestion of the enterotoxin formed in food during growth of certain strains of *Staphylococcus aureus*. Both octyl and dodecyl gallates were previously described to be effective against *Staph. aureus*, including methicillin-resistant *Staph. aureus* (MRSA) strains (*20*).

Alkyl gallates are known to induce cellular reactive oxygen species (ROS) generation (*21*). Membrane lipids are abundant in unsaturated fatty acids. The oxidation of these unsaturated fatty acids leads to a decrease in the membrane fluidity and disruption of membrane structure and function. Hence, ROS generation may explain alkyl gallates' bactericidal action. However, ROS generation in *S. choleraesuis* and *Staph. aureus* cells caused by octyl and dodecyl gallates is not directly associated with their bactericidal action, since the antioxidants such as α-tocopherol, L-ascorbate, and *N*-acetylcysteine did not exhibit a protective effect. Rather, these alkyl gallates acted as antioxidants and protected against oxidative damage.

Propyl, octyl, and dodecyl gallates are currently permitted for use as antioxidant additives in food (*22*). In fact, all the gallates, regardless of their alkyl chain length, showed potent scavenging activity on DPPH radicals (*23*). Since this indicates that the alkyl chain length was not directly related to this activity, all the gallate esters can be used as antioxidants. In addition to their potent antioxidant activity, amphiphilic alkyl gallates' broad antimicrobial activity would appear to be of great overall value. After the gallates are consumed together with the foods to which they are added as additives, these gallates are hydrolyzed to the original gallic acid and the corresponding alcohols, both of which are common in many edible plants. More importantly, the freed gallic acid still acts as a potent antioxidant. This concept has been further extended to geranyl gallate (**9**, **Figure 1**), since geraniol is reported to increase glutathione *S*-transferase activity, which is believed to be a major mechanism for chemical carcinogen detoxification (*24*). As expected, geranyl gallate exhibited activity against *S. choleraesuis*, with a MBC of 50 μg/mL. In this connection, geraniol is known in

a large number (>160) of essential oils—such as lemon grass, coriander, lavender, and carrot—and is used as food flavoring for baked goods, soft and hard candy, gelatin and pudding, and chewing gum. The antioxidant gallic acid and the glutathion *S*-transferase inducer geraniol may contribute to reduce cancer risk as well as oxidative damage-related diseases.

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## LITERATURE CITED

(1) White, R.; Jobling, S.; Hoare, S. A.; Sumpter, J. P.; Parker, M. G. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **1994**, *135*, 175−182.

(2) Soto, A. M.; Justicia, H.; Wray, J. W.; Sonnenschein, C. *p*-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environ. Health Perspect.* **1991**, *92*, 167−173.

(3) Neu, H. C. The crisis in antibiotic resistance. *Science* **1992**, *257*, 1064−1073.

(4) Davies, J. Inactivation of antibiotics and the dissemination of resistance genes. *Science* **1994**, *264*, 375−382.

(5) Shaw, K. J.; Rather, P. N.; Hare, R. S.; Miller, G. H. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol. Rev.* **1993**, *57*, 138−163.

(6) Spratt, B. G. Resistance to antibiotics mediated by target alterations. *Science* **1994**, *264*, 388−393.

(7) Kubo, I.; Xiao, P.; Fujita, K. Antifungal activity of octyl gallate: Structural criteria and mode of action. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 347−350.

(8) Frazier, W. C.; Westhoff, D. C. *Food Microbiology*; McGraw-Hill: New York, 1988; pp 39−58.

(9) Kubo, I.; Muroi, H.; Himejima, M.; Kubo, A. Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorg. Med. Chem.* **1995**, *3*, 873−880.

(10) Haraguchi, H.; Hamatani, Y.; Shibata, K.; Hashimoto, K. An inhibitor of acetolactate synthase from a microbe. *Biosci. Biotech. Biochem.* **1992**, *56*, 2085−2086.

(11) Imai, K.; Asano, A.; Sato, R. Oxidative phosphorylation in *Micrococcus denitrificans*. *Biochim. Biophys. Acta* **1967**, *143*, 462−476.

(12) Dancy, G. F.; Levine, A. E.; Shapiro, B. M. The NADH dehydrogenase of the respiratory chain of *Escherichia coli*. *J. Biol. Chem.* **1976**, *251*, 5911−5920.

(13) Toda, S.; Kumura, M.; Ohnishi, M. Effects of phenolcarboxylic acids on superoxide anion. *Planta Med.* **1991**, *57*, 8−10.

(14) Hansch, C.; Dunn, W. J., III. Linear relationships between lipophilic character and biological activity of drugs. *J. Pharm. Sci.* **1972**, *61*, 1−19.

(15) Haraguchi, H.; Oike, S.; Muroi, H.; Kubo, I. Mode of antibacterial action of totarol, a diterpene from *Podocarpus nagi*. *Planta Med.* **1996**, *62*, 122−125.

(16) Haraguchi, H.; Kataoka, S.; Okamoto, S.; Hanafi, M.; Shibata, K. Antimicrobial triterpenes from *Ilex integra* and the mechanism of antifungal action. *Phytother. Res.* **1999**, *13*, 151−156.

(17) Franks, N. P.; Lieb, W. R. Partitioning of long-chain alcohols into lipid bilayers: Implications for mechanisms of general anesthesia. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5116−5120.

(18) Miller, K. W.; Firestone, L. L.; Alifimoff, J. K.; Streicher, P. Nonanesthetic alcohols dissolve in synaptic membranes without perturbing their lipids. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1084−1087.

(19) Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 383−388.

(20) Kubo, I.; Xiao, P.; Fujita, K. Anti-MRSA activity of alkyl gallates. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 113−116.

(21) Nakayama, T.; Hiramitsu, M.; Osawa, T.; Kawakishi, S. The protective role of gallic acid esters in bacterial cytotoxicity and SOS responses induced by hydrogen peroxide. *Mutat Res.* **1993**, *303*, 29−34.

(22) Aruoma, O. I.; Murcia, A.; Butler, J.; Halliwell, B. Evaluation of the antioxidant and prooxidant actions of gallic acid and its derivatives. *J. Agric. Food Chem.* **1993**, *41*, 1880−1885.

(23) Kubo, I. Molecular design of antioxidative and antimicrobial agents. *Chemtech* **1999**, *29*, 37−42.

(24) Zheng, G. Q.; Kenney, P. M.; Lam, L. K. Potential anticarcinogenic natural products isolated from lemongrass oil and galanga root oil. *J. Agric. Food Chem.* **1993**, *41*, 153−156.

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