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# Model Chlorination of Plant Derived Phenolic Water Contaminants with an Assessment of Their Potential Toxicity to *Escherichia coli*

J. T. Borlakoglu,[1,*] and R. Kickuth[2]

[1]University of Reading, Department of Physiology and Biochemistry,
Whiteknights, P.O. Box 228, Reading RG6 2AJ, England and [2]University of
Kassell, Lehrstuhl für Ökochemie, 3430 Witzenhausen, West Germany

As a safety measure drinking water is often chemically chlorinated to prevent water-transmittable epidemic diseases. However, great concern about routine water chlorination has been expressed, due to the possible chlorination of unwanted aliphatic and aromatic water contaminants. Phenolic compounds are potentially hazardous and are known to be an important group of aromatic water contaminants (Kraybill 1978). After chlorination, they are likely to change their chemical and biological behaviour with the synthesis of unstable and stable organochlorine derivatives (Tardiff et al 1978). Although industrial and agricultural chemicals are the major contributors to organic water contamination the importance of plant derived phenolic compounds has not yet been fully appreciated.

During the annual litter decay a number of phenolic compounds are released into the environment. Previous studies on water courses have shown that five phenolic acids derived from plants predominate, with 4-hydroxycinnamic acid as the major compound (Kickuth 1982). After water chlorination these compounds are likely to become chlorinated with the production of stable organochlorine derivatives.

The objective of the present study was to assess the effects of chlorination on seven plant-derived phenolic compounds. The substitution pattern of the parent (non-chlorinated) compounds was correlated to the number of produced chlorophenolic derivatives (Table 1) (structure versus chemical reactivity). The formed chlorophenolic mixtures were applied to Escherichia coli DSM 613 to examine their potential toxic effects. Escherichia coli DSM 613 was used as an indicator of toxicity for environmental pollution.

## MATERIALS AND METHODS

Currently, we described the chlorophenolic synthesis and analysis of single chlorophenolic derivatives (Borlakoglu and Kickuth 1986).

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*Author to whom correspondence should be sent

Escherichia coli DSM 613 was cultured overnight in a medium containing 3.0 g meat extract and 5.0 g pepton exchange (Firma Merck, 5443 Darmstadt, West Germany).

Agar plates from Firma Merck, 7881 appeared to be advantageous containing

| | |
|---|---|
| Special pepton | 15, 6 g |
| Yeast extract | 2, 8 g |
| NaCl | 5, 6 g |
| Glucose ($D^+$) | 1, 0 g |
| Agar-agar | 12, 0 g |

The mixture was dissolved in 1 l aquadest. With a small gimlet round holes of 0.2 cm diameter were drilled into the agar plates and small cellulose plates containing the chlorophenolic mixture were placed into it. The chlorophenols were previously dissolved in hot glycerine.

The cells were in steady state and incubated at 37°C under optimum conditions. Bacterial toxicity was measured as inhibition of growth in cm at various concentrations. For graphic illustrations concentrations were plotted as $\log_{(10)}$ of ppm chlorophenolic mixture. Each point represents at least 5 replicates and is expressed as the arithmetic mean.

## RESULTS AND DISCUSSION

The chlorophenolic synthesis of plant-derived aromatics produced a large number of derivatives and the relationship between structure of the parent compound and the number of produced chlorophenolic derivatives is shown in Table 1.

Table 1 Substitution pattern of the parent (non-chlorinated) compound and the number of produced chlorophenolic derivatives.

| Compounds | Number of chlorophenolic derivatives detected on a 1-dimensional TLC at 254 nm | Number of chorophenolic derivatives after addition of OH-group in position C-3 of the aromatic ring | Number of chlorophenolic derivatives after replacement of OH-group in position C-3 with $OCH_3$-group |
|---|---|---|---|
| 4-hydroxybenzoic acid | 2 | 10 (+8)* | 15 (+13)* |
| 4-hydroxybenzaldehyde | 6 | - | 13 (+7)* |
| 4-hydroxycinnamic acid | 8 | - | 11 (+3)* |

*Figures in brackets represent the increase in the number of derivatives

The substitution and hydroxylation pattern of the parent (non-chlorinated) compound influenced the reactivity of the chlorophenolic synthesis and the number of produced chlorophenolic derivatives. Vicinal substitution of the aromatic ring is linked to a larger number of derivatives compared to para-substitution. The C-3/C-4 dihydroxy substitution pattern of the aromatic ring favoured the synthesis of multiple derivatives as in the case of 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid. Both phenolics produced 10 and 13 chloro-derivatives respectively. Additionally, in the case of 3,4 dihydroxybenzoic acid the replacement of C-3-hydroxyl group with a methoxy group enhanced the number of produced chloro-derivatives from 8 to 13. Probably the formation of phenolic radicals during chlorination determines the reactivity of the synthesis and the number of produced unstable and stable chlorophenolic derivatives. Especially the C-3/C-4 vicinal substitution type appears to be highly reactive with the formation of a large number of derivatives compared to para-substituted phenolics.

After chlorination, 4-hydroxybenzaldehyde produced four additional chloro-derivatives compared to 4-hydroxybenzoic acid. However, 4-hydrobenzoic acid appears to be highly reactive after substitution with a hydroxyl or methoxy group. This was certainly unexpected, because the parent 4-hydroxybenzoic acid produced after chlorination only two chlorophenolic derivatives compared to 10 derivatives with a C-3 hydroxyl group and 15 derivatives with a C-3 methoxy group. For both risk assessment and chlorophenolic synthesis the presence of a C-3- hydroxyl or C-3 methoxy group is of great importance. In the case of 4-hydroxybenzoic acid the addition of an OH-group in position C-3 increased the number of chlorophenolic derivatives 4-fold and the replacement of the OH-group with an $OCH_3$-group increased the number of chlorophenolic derivatives 7.5-fold. The occurrence of a methoxy group in position C-3 enhanced the number of chlorophenolic derivatives in 4-hydroxybenzaladehyde 2.2-fold and in 4-hydroxycinnamic acid 1.4-fold.

With the aid of these model reactions it was possible to evaluate the potential synthetic event of water chlorination in the presence of plant-derived phenolic compounds. The present findings indicate the formation of > 65 stable chlorophenolic derivatives after chlorination of 7 naturally occurring phenolics. However, although many of these compounds are likely to be acceptable in low concentrations with the formation of such a large number of derivatives the accumulative concentration is relatively high. Moreover it has yet to be proven that individual derivatives are important toxicants or whether there are synergistic effects when they are applied in combination with other chloroderivatives and water contaminants. This could lead to an unacceptable level of toxicity.

We were able to identify the major plant-derived chlorophenolic derivative of 4-hydroxycinnamic acid and have published the proton nuclear magnetic resonance-, infrared- and ultraviolet spectra including the elementary analysis (Borlakoglu and Kickuth 1986). This compound is identified as 3,5 dichloro-4-hydroxyphenyl-αβ-dichloropropionic acid (Fig. 1). Up to 80% of the chlorophenolic synthesis of 4-hydroxycinnamic was yielded as this major chlorophenolic derivative. Moreover, under the described synthetic conditions the chlorination of 4-hydroxybenzoic acid led to the formation of 3,5-dichloro-4-hydroxybenzoic acid which was confirmed by spectral and elementary analysis.

However, many of the 65 formed stable chlorophenolic derivatives are quantitatively very small indeed and it is therefore difficult to get enough synthetic material to elucidate their chemical structure and absolute configuration. Whilst the authors believe in the importance of identifying the chemical structures of all 65 chlorophenolic derivatives, the present study aims to assess the toxic potential of the formed mixtures. This is very important since these compounds, when formed, appear as mixtures rather than single chloro derivatives. Under in vivo conditions it is most likely to be exposed to a complex mixture of all formed chlorophenolic derivatives. Therefore, in order to mimic their occurrences in water supplies any toxicological assessment has to be done on the extracted mixtures.

We chose E. coli as a test organism and indicator for environmental pollution.

Table 2 Toxic concentrations of plant derived chlorophenolic derivatives on E. coli DSM 613.

| Chlorophenolic mixture | Inhibition of E. coli growth |
|---|---|
| 4-hydroxybenzaldehyde | 312 ppm |
| 4-hydroxybenzoic acid | > 5000 ppm* |
| 3,4-dihydroxybenzoic acid | 156 ppm |
| 4-hydroxy-3-methoxy-benzaldehyde | 312 ppm |
| 4-hydroxy-3-methoxybenzoic acid | 625 ppm |
| 4-hydroxycinnamic acid | 1250 ppm |
| 4-hydroxy-3-methoxycinnamic acid | 1250 ppm |

*Has not found to be toxic even at the concentration indicated.

As can be seen from Table 2 there are remarkable differences in the inhibition of bacterial growth ranging from 156 ppm to 1250 ppm (see also Figs.2-6). In the case of the chlorophenolic mixture of 4-hydroxybenzaldehyde the additional substitution of a methoxy group in position C-3 had no effect on the toxic potential, although the methoxy substitution increased the number of produced chlorophenolic derivatives from 6 to 13. The same

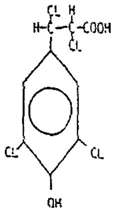

Figure 1. 3,4 Dichloro-4-hydroxyphenyl-α,β dichloropropionic acid.

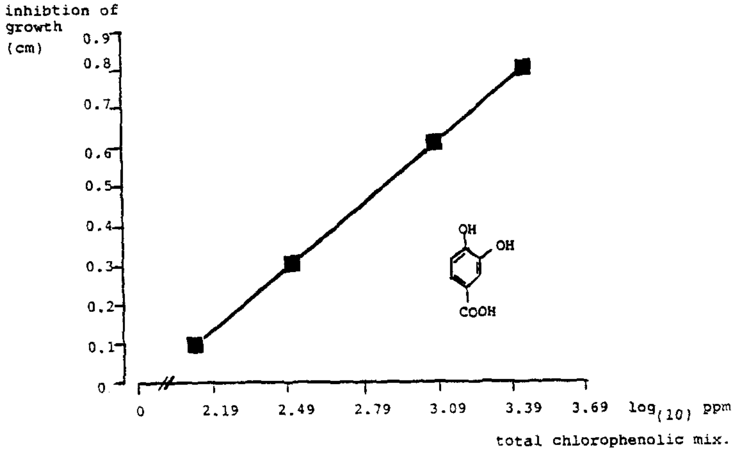

Figure 2. Growth inhibition of E.coli DSM 613 with the chlorophenolic mixture of 3,4-dihydroxybenzoic acid.

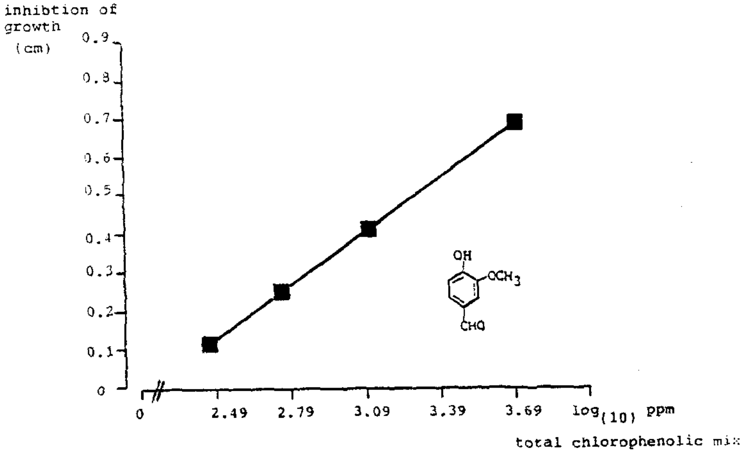

Figure 3. Growth inhibition of E.coli DSM 613 with the chlorophenolic mixture of 3-methoxy-4-hydroxy-benzaldehyde.

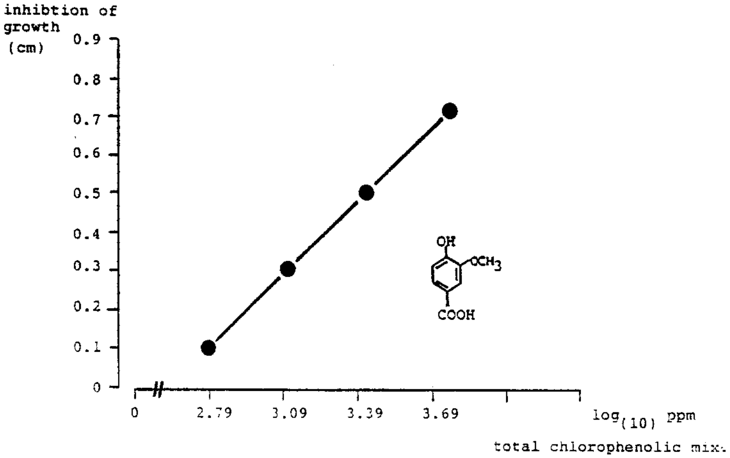

Figure 4. Growth inhibition of E.coli DSM 613 with the chlorophenolic mixture of 3-methoxy-4-hydroxy-benzoic acid.

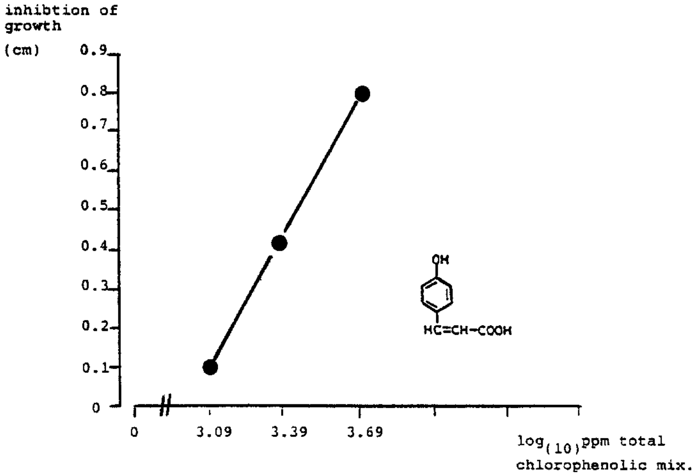

Figure 5. Growth inhibition of E.coli DSM 613 with the chlorophenolic mixture of 4-hydroxycinnamic acid.

pattern applies to the chlorophenolic mixture of 4-hydroxycinnamic acid, which were only toxic at 1250 ppm. Although the additional methoxy group in position C-3 had no further effect on the toxic potential, the number of chlorophenolic derivatives increased from 8 to 11. The chlorophenolic mixture of 4-hydroxybenzoic acid did not produce any inhibition of E. coli growth even at a concentration of > 5000 ppm. Obviously the synthesis of chlorobenzoic acid derivatives had no effect on bacterial growth. Other workers could show similar results for 4-hydroxybenzoic acid on Pseudomonas (Koppe et al 1982). By

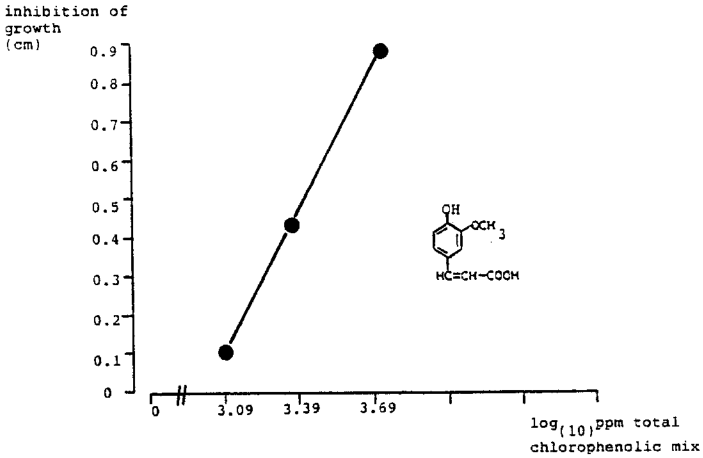

Figure 6. Growth inhibition of E.coli DSM 613 with the chlorophenolic mixture of 3-methoxy 4-hydroxycinnamic acid.

comparison, the chlorophenolic mixture of 3,4 dihydroxybenzoic acid appeared to be highly toxic, causing inhibition of growth at a concentation of 156 ppm. The replacement of the hydroxyl group in position C-3 with a methoxy group decreased the toxic potential of the chlorophenolic mixture by 100%. This result contrasts the findings of the other chlorophenolic compounds studied.

Because of its high toxicity, 3,4-dihydroxybenzoic acid has been found to be most important compared to the other chemicals investigated. The additional hydroxylation in position C-3 of 4-hydroxybenzoic acid had a strong impact in producing both a large number of chloroderivatives and the most toxic compound studied. It appears that the hydroxylation pattern of the aromatic system is linked to the toxic potential of the compound, but also reinforces the production of a large number of chlorophenolic derivatives (see Table 2). In anology, Flood and Kirkham (1960) could show similar effects on Venturia inaequalis and Venturia pirinia. They demonstrated that 4-hydroxycinnamic acid and 3-methoxy-4-hydroxycinnamic acid did not produce any inhibition of growth, whereas 3,4-dihydroxybenzoic acid and 3,4-dihydroxycinnamic acid caused remarkable inhibition. The authors believe that the vicinal hydroxylation of the aromatic system is an important requirement in producing toxic chlorophenolic derivatives.

Phenolic compounds are an important group of environmental pollutants and water contaminants. Depending on the pH of the solution they have a high partition coefficient for lipids and hydrophobic compounds and are known to be membrane poisoning (Riley and Seal 1974). In the present study a bacterial system was used to evaluate the potential toxic effects of plant-derived chlorophenolic mixtures to E. coli as a test organism. Although the bacterial cell wall differs from the mammalian cell membrane the authors assume possible toxic effects, in humans, if such chlorophenolic mixtures are consumed. Therefore future work must incorporate studies on the mammalian intestine. This would provide important information about the toxicological potential of plant-derived phenolic compounds and would help to evaluate the risk of plant-derived phenolic water contamination.

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