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In Vitro Antioxidant Activity and Antigenotoxicity of 5-*n*-Alkylresorcinols

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Incubation with 5-*n*-alkylresorcinols (chain lengths C15:0, C17:0, C19:0, C21:0, and C23:0) increased the self-protection capacity of HT29 human colon cancer cells against DNA damage induced by hydrogen peroxide and genotoxic fecal water samples using comet assay (single-cell gel electrophoresis assay). The alkylresorcinols did not exert potent antioxidant activity in the FRAP (ferric reduction ability of plasma) and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assays. However they were able to significantly inhibit copper-mediated oxidation of human LDL (low-density lipoprotein) in vitro, and pentadecylresorcinol at 25 μmol/L increased lag time by 65 min. The results show that alkylresorcinols have antigenotoxic and antioxidant activity under in vitro conditions.

KEYWORDS: Alkylresorcinols; comet assay; colorectal cancer; LDL oxidation; antioxidants

INTRODUCTION

5-*n*-Alkylresorcinols are phenolic lipids that carry an *n*-alkyl side chain at position 5 of the resorcinol ring (**Figure 1**). These phytochemicals are present in the aleurone of cereal grains (rye, wheat, and triticale), the highest concentrations occurring in rye (*1*−*3*). The concentrations found vary from 300 mg/kg in whole wheat (*4*) to 3000 mg/kg in whole rye (*5*). Epidemiological and experimental studies suggest that whole grain products possess protective activity against cardiovascular diseases and some forms of cancer, especially colorectal and breast cancer (*6*, *7*). Oxidative and free radical reactions have been implicated in coronary heart disease and cancer (*8*, *9*), and dietary antioxidants are thought to reduce the risk of these diseases (*10*). The presence of hydroxyl groups suggests that alkylresorcinols have radical scavenging and hydrogen donation abilities. Previous research on their antioxidant properties includes studies on pentadecylresorcinol (C15:0), which has been found to slow the rate of oil and lipid oxidation (*11*). Pentadecylresorcinol was a stronger inhibitor of H_2O_2-induced oxidation of erythrocyte membranes than nona- or tridecylresorcinols (C19:0, C23:0) (*12*). The radical scavenging and hydrogen donation power of pentadecylresorcinol was low (*13*). Apparently due to their long alkyl chains, alkylresorcinols interact with biological membranes affecting e.g. the lipid bilayers (*14*) and the properties of the hydrophobic environment (*15*). Alk(en)-ylresorcinols and other related compounds such as diarylalkanes, dialkylresorcinols, oxoalkylresorcinols, and alkylresorcinol sulfates have various biological effects, e.g. antimutagenic activity (*16*−*19*), antibacterial properties (*20*), and inhibition of enzymes (*21*−*24*) which are under active study. Currently, the use of alkylresorcinols as biomarkers of whole grain intake is of great potential due to their near-unique presence in rye- and wheat-containing foods (*25*).

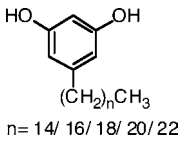

Figure 1. Structure of 5-*n*-alkylresorcinols present in rye.

The aim of this study was to investigate the biological activity of 5-*n*-alkylresorcinols present in rye (chain lengths C15:0, C17:0, C19:0, C21:0, and C23:0, **Figure 1**) by screening their ability to protect against DNA damage in the initiation step in carcinogenesis. For our cellular study, to measure the capacity of the alkylresorcinols to reduce DNA damage, we chose the HT29 cell line, which is widely used as a model for studying colon carcinogenesis in combination with the single cell gel electrophoresis (comet) assay (*17*, *26*). Hydrogen peroxide and fecal water (*27*, *28*) were used as the inducers of genotoxic damage. In vitro antioxidant activity was assessed by two chemical methods—FRAP (ferric reduction ability of plasma) assay to measure the antioxidant power by monitoring the reduction of a ferric tripyridyltriazine complex to its ferrous form and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assay, to screen the hydrogen donation ability. Furthermore, the ability of alkylresorcinols to prevent copper-mediated oxidation of human LDL (low-density lipoprotein) in vitro was studied, since limiting LDL oxidation might prevent the first stage in the development of atherosclerosis.

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MATERIALS AND METHODS

Alkylresorcinols. 5-*n*-Alkylresorcinols (C15:0, C17:0, C19:0, C21:0, and C23:0) were prepared in the Laboratory of Organic Chemistry, University of Helsinki, Finland. The purity and identification of the products were established by ^{1}H and ^{13}C NMR and mass spectrometry.

FRAP Assay. The FRAP assay was run according to a modification of the method of Benzie and Strain (*29*). Alkylresorcinol samples (C15:0, C17:0, C19:0, and C21:0; methanolic solutions) were tested at various concentrations (0, 250, 500, 750, and 1000 μmol/L). Duplicates were run for each concentration. The assay was run on a Hitachi 912 analyzer.

DPPH Assay. The assay was run according to the method described by Peterson et al. (*30*). Aliquots of alkylresorcinols (C15:0, C17:0, C19:0, and C21:0) were added to a DPPH solution and incubated for 3 h. Duplicates were run for each concentration of alkylresorcinols (0, 5, 10, and 15 μmol/L). The absorbance change compared to the blank sample at 515 nm (measured with Spectronic Genesys 2 spectrometer) was used to calculate the amount of DPPH reduced.

Inhibition of Low-Density Lipoprotein Oxidation. The test is based on the continuous monitoring of the formation of conjugated diene structures in the Cu(II)-induced oxidation process of LDL. LDL was isolated from human plasma by ultracentrifugation using a Beckman ultracentrifuge and Beckman NVT65 rotor (60 000 rpm, 7 °C, 2.5 h). Isolated LDL was dialyzed and stored under nitrogen atmosphere at 4 °C for no longer than 48 h. The experiment was carried out in a series of 1.5 mL quartz cuvettes. An appropriate amount of LDL (0.01 μmol/L in the final solution) and PBS (phosphate-buffered saline) were added to the cuvettes to make a final volume of 1 mL with the alkylresorcinol (C15:0 or C17:0) solution (50% methanolic solution, 100 μL) and cupric chloride solution (1000 μmol/L, 11.7 μL). To limit the amount of organic solvents used in the assay, the LDL oxidation experiments were run with penta- and heptadecylresorcinols only and less soluble longer chain alkylresorcinols were excluded. Alkylresorcinols were added to all except the control solutions, and duplicates were run for each concentration (0, 2.5 and 25 μmol/L). The 4 h experiment was run at 37 °C with a UV/vis spectrometer Philips PU8730 measuring the absorbance at 234 nm every 5 min. The length of the lag phase was defined as the time to the intercept of the tangent of the oxidation curve with baseline.

Tissue Culture. The HT29 cells were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, U.K.). Dulbeccos minimum essential medium (DMEM) was obtained from Sigma. HT29 cells were cultured in Roux flasks as monolayers in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were cultured for 7 days at 37 °C with filtered air containing 5% CO_2 before use in the tests, and the medium was changed every 2 days.

In Vitro Genotoxicity Studies (Comet Assay). The cell line HT29 which is widely used as a model for colon cancer was chosen for the study. The HT29 cells were preincubated at 37 °C (1 or 24 h) with different alkylresorcinols at various concentrations (0, 10, and 100 μmol/L). Alkylresorcinol solutions were made in DMSO (dimethyl sulfoxide) and diluted with DMEM (final percentage of DMSO, 0.5%). After incubation, cells were harvested, washed with PBS, and resuspended by the addition of trypsin (0.25% trypsin−EDTA (ethylenediaminetetraacetic acid)) at 37 °C for 5 min. A cell and viability count was performed using a Coulter counter and trypan blue dye (Sigma, Poole, U.K.). Viability was ≥98%. The cells were centrifuged at 285*g* for 3 min and resuspended in DMEM medium.

The comet assay was performed essentially as described by Venturi et al (*28*). The cell suspensions (preexposed to alkylresorcinols at 0, 10, and 100 μmol/L) were challenged with hydrogen peroxide (75 μmol/L, 5 min, 4 °C). A more limited study was conducted on cells preexposed to 0 or 50 μmol/L concentrations of alkylresorcinols and challenged with fecal water (50 μL + 450 μL cell suspension, 30 min, 37 °C), which was obtained by high-speed centrifugation (50000*g* for 2 h at 10 °C) of a homogenized fecal sample from a healthy subject. The sample was previously shown to have genotoxic activity toward HT29 cells. After the period of challenge with the genotoxic agent, the cell suspension was centrifuged for 5 min at 285*g*. The supernatant was discarded and the cell pellet resuspended in 85 μL of 0.85% low melting point agarose in PBS. The suspension was added to previously prepared normal melting point agarose gels (1% in PBS) on fully frosted slides, and cover slips were added. The slides were immersed in lysis buffer (2.5 M NaCl, 100 μM Na_2EDTA, 10 mM TRIS, pH 10) for at least 1 h at 4 °C and then placed in electrophoresis buffer and allowed to unwind for 20 min. The electrophoresis was run at 26 V and 300 mA for 20 min. After the electrophoresis, the gels were washed with neutralization buffer (0.4 M TRIS, pH 7.5, 5 min wash) three times at 4 °C. Gels were stained with 35 μL of ethyl bromide solution (0.05 mg/mL) and stored under a moist atmosphere at 4 °C for no longer than 48 h before scoring.

Images were analyzed at 400× magnification using a fluorescence microscope. From each slide, 50 randomly selected cells were measured and a percentage of tail DNA was recorded using Komet 5.0 image analysis software (Kinetic Imaging Ltd., Liverpool, U.K.). Positive (hydrogen peroxide, 75 μmol/L, or fecal water) and negative (PBS) controls were included for every experiment. The mean percentage of tail DNA was calculated from 50 cells/gel (each sample in triplicate). Standard deviation and standard error of the mean (SEM) were estimated for each experiment. The significance of the results was estimated by analyzing them statistically by Dunnett's test.

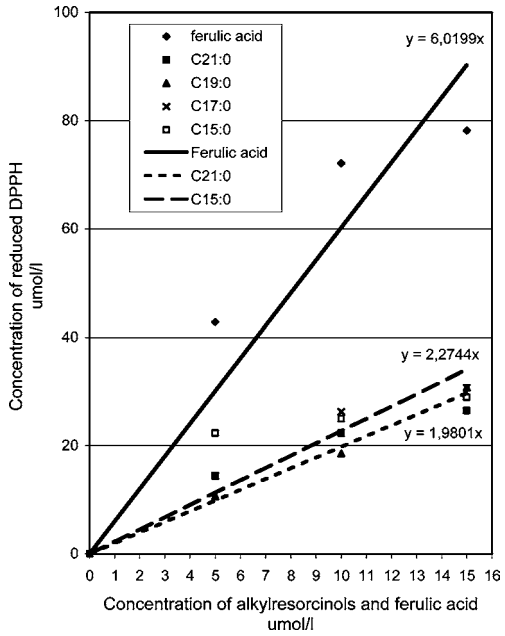

Figure 2. Antioxidant activity of alkylresorcinols compared to the activity of ferulic acid by DPPH assay. Concentrations are given in μmol/L. Data points represent averages from duplicate assays that agreed within an average of 7.2%. Trend lines clarify the results of longer and shorter chain alkylresorcinols (C21:0 and C15:0) and ferulic acid.

RESULTS

In the FRAP assay alkylresorcinols gave a linear, dose-related response but had only ca. 10% of the activity of ferulic acid, which is a commonly occurring antioxidant in cereals (data not shown). Similarly, alkylresorcinols showed relatively low antioxidant activity in the DPPH assay (**Figure 2**). However the results of the inhibition of LDL oxidation showed that alkylresorcinols possessed antioxidant properties in a biological system. Pentadecylresorcinol at 25 μmol/L concentration increased the lag time by 65 min (**Figure 3**).

The antigenotoxic effect of alkylresorcinols on hydrogen peroxide-induced DNA damage in HT29 cells following 1 or 24 h incubation periods was stronger in the 24 h experiments (**Figures 4** and **5**). Penta- and heptadecylresorcinols (C15:0 and

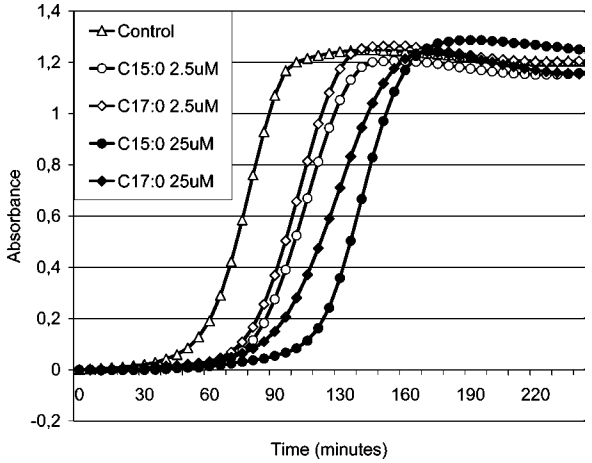

Figure 3. Effect of alkylresorcinols (C15:0 and C17:0) on Cu(II)-mediated LDL oxidation. The absorbance of conjugated dienes is given as a function of time. Data points represent averages from duplicate assays. The solvent control curve (5% methanol) is not shown but did not differ from the control.

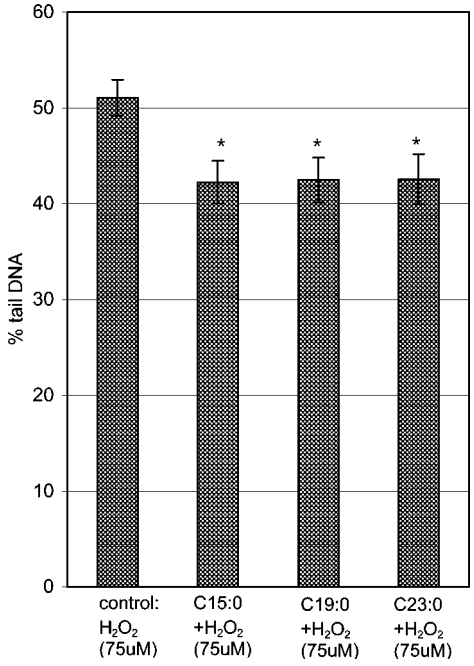

Figure 4. Effect of 1 h incubation of alkylresorcinols (C15:0, C19:0, and C23:0) at 100 μmol/L concentration on the H_2O_2 treated HT29 cells DNA damage (% tail DNA) by comet assay. Values are the average percentage of tail DNA ± SEM. Three independent experiments were run. The asterisks represent the significance (*, $P < 0.05$) compared with the control.

C17:0) gave the most significant results ($P < 0.005$) at a 100 μmol/L concentration, where also a response to the chain length was shown (**Figure 5**). The decrease in genotoxicity at this concentration was approximately 40% with pentadecylresorcinol and 10% with heneicosylresorcinol (C21:0). In comparison, at a concentration of 10 μmol/L the effect of alkylresorcinols averaged a decrease of only 20%. The addition of alkylresorcinols to the cells did not cause any increase in DNA damage compared to PBS (negative control). However, pentadecylresorcinol at 100 μmol/L was found to be cytotoxic in the 24 h preincubation experiment, with only 10% survival vs the control incubations.

The effect of alkylresorcinols at a 50 μmol/L concentration on the DNA damage of the HT29 cells exposed to fecal water treatment is shown in **Figure 6**. The decrease in genotoxicity

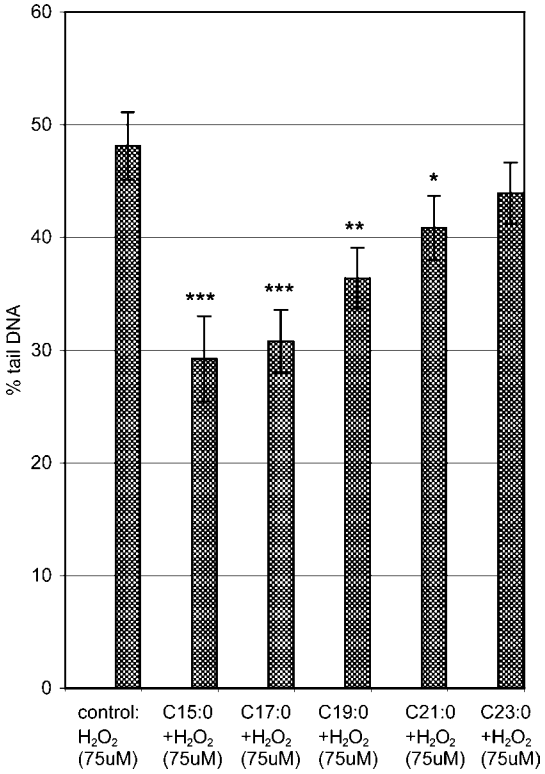

Figure 5. Effect of 24 h incubation of alkylresorcinols (C15:0, C17:0, C19:0, C21:0, and C23:0) at 100 μmol/L concentration on the H_2O_2 treated HT29 cells DNA damage (% tail DNA) by comet assay. Values are the average percentage of tail DNA ± SEM. Three independent experiments were run. The asterisks represent the significance (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$) compared with the control.

was significant ($P < 0.01$) with pentadecylresorcinol, which reduced the damage by 40%.

DISCUSSION

Human studies support the experimentally based notion of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor (*31*). The H_2O_2 challenge has been used in a number of studies to examine the effect of vegetable extracts for this reason (*32*, *33*). The results of our study show that alkylresorcinols have antigenotoxic effects toward oxidative damage induced by hydrogen peroxide challenge in the human colon cancer cell line HT29. Since the alkylresorcinols were removed from the cells before exposure to the H_2O_2 challenge, the reduction of induced DNA damage indicates an increased cellular capacity to protect against oxidative damage.

It is of importance that also the genotoxicity of fecal water was reduced, since this may have direct relevance to colorectal cancer risk. We have demonstrated, using the comet assay, that approximately 30% of all fecal water samples from human volunteers are highly genotoxic toward a human colon cell line (*28*). Since mutations and other genetic changes are critical factors in the initiation and development of colorectal cancer (*34*), this observation is of potentially great significance for the etiology of the disease. The use of fecal water in conjunction with the comet assay and human colon cell lines provides a useful and highly relevant in vitro model to investigate dietary components for potential anticancer activity in the colon.

The mechanism of the antigenotoxicity of alkylresorcinols is not clear. If direct antioxidant effects are considered, the compounds did not show high antioxidant activity in the

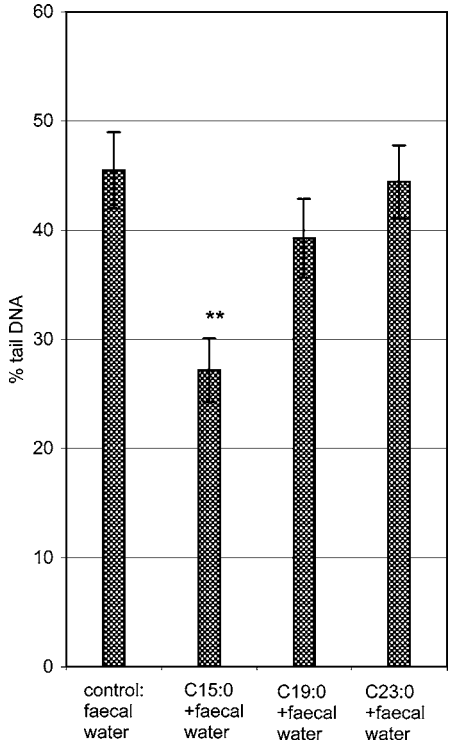

Figure 6. Effect of 24 h incubation of alkylresorcinols (C15:0, C19:0, and C23:0) at 50 μmol/L concentration on the fecal water treated HT29 cells DNA damage (% tail DNA) by comet assay. Values are the average percentage of tail DNA ± SEM. Three independent experiments were run. The asterisks represent the significance (**, $P < 0.01$) compared with the control.

FRAP and DPPH assays but had a strong protective effect against Cu-induced LDL oxidation. The effects therefore might be based on the ability of the compounds to interact with biological membranes. Limiting LDL oxidation might prevent the first stage in the development of atherosclerosis and this thus provides a potential mechanism to explain the observed protective activity of whole grain cereals toward cardiovascular disease in epidemiological and experimental studies.

The actions of alkylresorcinols in vivo depend on their absorption and metabolism in the body. They are partly absorbed or metabolized in the small intestine (*35*) and they have been detected from human plasma, at a concentration of approximately 0.35 μmol/L (*36*). The lowest required concentrations of alkylresorcinols to significantly reduce the oxidative DNA damage in our in vitro studies were found to be 30 times higher than the concentrations determined from plasma. It should be noted however that tissue concentrations may be higher than those in plasma. In the case of isoflavones for example, the concentration in prostate tissue has been shown to be as much as 10 times higher than the plasma level (*37*). The biological activity of the alkylresorcinols in the assays used in the present study appeared to be dependent on chain length. This may be a consequence of solubility, which is limited because of the extended nonpolar alkyl chain. The solubility decreases with increasing chain length.

The results show that alkylresorcinols have antigenotoxic and antioxidant activity in biological systems under in vitro conditions. However further in vitro and in vivo work is needed to evaluate their physiological significance.

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