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Preclinical Evaluation of Rapeseed, Raspberry, and Pine Bark Phenolics for Health Related Effects

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Rapeseed, raspberry, and pine bark are promising bioactive sources of plant phenolics selected from among ca. 100 previously screened plant materials for in vitro preclinical evaluation of health related effects. Phenolic extracts and isolated fractions of the selected materials were investigated for antioxidant, antimicrobial, antiinflammatory, and antimutagenic properties as well as for cell permeability. It was shown that rapeseed and pine bark phenolics and raspberry anthocyanins were good or excellent antioxidants toward oxidation of phosphatidylcholine membrane (liposomes), rapeseed oil (crude) phenolics were effective radical scavengers (DPPH test), and both raspberry and pine bark phenolics inhibited LDL oxidation. Rapeseed oil phenolics, principally vinylsyringol, raspberry anthocyanins, and pinoresinol and matairesinol, the principal components of pine bark phenolic isolate, were effective against formation of the proinflammatory mediator, prostaglandin E_2. Raspberry ellagitannins inhibited the growth of *Proteus mirabilis* and *Klebsiella oxytoca*. Pine bark and rapeseed had minor effects on the permeability of model drugs in Caco-2 experiments. None of the tested extracts were mutagenic nor toxic to Caco-2 cells or macrophages. Thus, phenolic isolates from rapeseed, raspberry, and pine bark and are safe and bioactive for possible food applications including functional foods intended for health benefit.

KEYWORDS: Bioactivity tests; raspberry; pine bark and rapeseed phenolics

INTRODUCTION

Phenolic compounds exist widely in plants. They are plant secondary metabolites, and they have an important role as defense compounds. The exact role of these secondary metabolites is still unclear, but it is known that phenolic compounds are important in the survival of a plant in its environment (*1*). In addition to benefits for plants, they have several properties beneficial to plant products for humans use. Several plant-derived medicines, which can prevent or cure some diseases, are rich in phenolic compounds (*2*). Phenolic compounds have been shown to possess positive effects on human health, for example protection against coronary heart disease and carcinogenesis (*3*). They can be classified into the following subgroups: phenolic acids, flavonoids, isoflavonoids, lignans, stilbenes, and complex phenolic polymers (*4*).

In our previous studies, ca. 100 phenolic extracts from different edible and nonedible plant materials were screened for antioxidant activities and antimicrobial effect (*5*−*7*). Among the most promising bioactive plant materials were pine bark, raspberry, and rapeseed.

Pine bark extract was recently shown to have antiinflammatory activity in inhibiting the production of two antiinflammatory mediators, nitric oxide and prostaglandin E_2 (*8*). Kähkönen et

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Table 1. The Phenolic Composition (%) of the Main Phenolics Identified in Rapeseed, Raspberry, and Pine Bark Phenolic Extracts and Fractions

extract	phenolic composition	ref
raspberry extract	51% ellagitannins, 31% anthocyanins	*12*
raspberry ellagitannin fraction	95% purity, the main compounds sanguiin H6 and lambertianin C	*17*
raspberry antocyanin fraction	60% cyanidin-3-sophoroside, 16% cyanidin-3-glucoside, 15% cyanidin-3-glycosyl-rutinoside, 5% cyanidin-3-rutinoside, 2% pelargonidin-3-rutinoside	*30*
rapeseed meal extract	64% sinapic acid, 4% sinapine	*7*
rapeseed oil extract	87% vinylsyringol, 7% sinapine, 6% sinapic acid	*21*
pine bark extract	34% procyanidins (oligomers), 33% catechins and catechin derivatives, 7% taxifolins and taxifolin derivatives, 9% lignan glucosides, 9% phenolic acid glucosides	*8*
pine bark chloroform fraction	23% β-hydroxypropiovanillone, 9% vanillin, 38% dihydroconeferyl alcohol, 14% ferulic acid, 5% pinoresinol, 10% matairesinol	*8*
pine bark semipreparative fraction	47% ferulic acid, 19% pinoresinol, 34% matairesinol	*8*

al. (*5*) tested the antioxidant activity of pine bark extract in a methyl linoleate model system, and Rauha et al. (*6*) tested the antimicrobial activity of pine bark. Laitinen et al. (*9*) showed that pine bark extract decreased the permeation of the drug compounds verapamil and metoprolol in the Caco-2 cell model. The main phenolic compounds in pine bark extract are phenolic acid glucosides, catechins and catechin derivatives, taxifolin and taxifolin derivatives, lignan glucosides, and procyanidins (**Table 1**) (*8, 10, 11*).

Raspberry phenolics are effective antioxidants toward oxidation of methyl linoleate (*5*), liposome membranes with incorporated lactalbumin (*12*), and an LDL model system (*13*). The antioxidant activity of raspberry phenolics has also been investigated in total oxyradical scavenging capacity assay (*14*) and using electron spin resonance spectroscopy (*15*). In addition, raspberry phenolics exhibit antiproliferative activities (*14*) and vasorelaxation properties (16). The main phenolics of raspberry are ellagitannins and anthocyanins (**Table 1**) (*12*). The main anthocyanin compound in raspberry is cyanidin-3-sophoroside, and the main ellagitannin compounds are sanguiin H-6 and lambertianin C (*15, 17*).

Rapeseed phenolics obtained from the oil pressing meal residue have shown antioxidant activity toward oxidation of LDL and liposomes (*7*), β-carotene (*18*), lipid emulsion (*19*), and methyl linoleate model systems (*20*). Rapeseed meal phenolics are effective as radical scavengers (*7*) and possess antimicrobial activities (*19*). The phenolic compounds in crude rapeseed oil are effective toward oxidation of lecithin-liposomes (*20*). Nowak et al. (*19*) showed that rapeseed meal phenolics have also antimicrobial activities. The main phenolic compounds in rapeseed are sinapine, the choline ester of sinapic acid, and sinapic acid (*7*). In crude postexpelled rapeseed oil vinylsyringol, a decarboxylation product of sinapic acid, is the main phenolic compound, followed by sinapine and sinapic acid (**Table 1**) (*21*).

Rapeseed, pine bark, and raspberry were selected for in vitro preclinical evaluation of health related effects on the basis of their promising bioactivities screened in our previous studies (*5*−*7*). The aim of this study was to investigate the antioxidant, antimicrobial, antiinflammatory, and antimutagenic properties as well as cell permeability of the phenolic extracts and isolated fractions of these selected materials. Some of the results have been published elsewhere concerning LDL oxidation of rapeseed (*7*), antiinflammatory effects of pine bark (*8*), and antimutagenic and cell permeability effects of raspberry (*22*).

MATERIALS AND METHODS

Chemicals and Reference Materials. *Chemicals.* 1,1-Diphenyl-2-picrylhydrazyl (DPPH) reagent was from Extrasynthèse (Genay, France). Methanol was HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Acetone (analytical grade) was purchased from Riedel-de Haen (Seelze, Germany), acetonitrile (HPLC grade) and chloroform (analytical grade) were purchased from Lab-Scan (Dublin, Ireland), and ethanol (99.5% v/v) was from Primalco (Rajamäki, Finland), formic acid (98−100%) from Fischer Scientific (Loughrough, U.K.), and *n*-hexane (HPLC grade) from Mallinckrodt (Denventer, Holland). MilliQ (Millipore, Bedford, MA) and Elgastat UHQ-PS (Elga, Kaarst, Germany) water systems were used. Ammonium dihydrogen phosphate, sodium diphosphate, disodium phosphate, cupric acetate, copper sulfate, and sodium chloride were from Merck, Germany. Lecithin from soybean (containing 40% phosphatidylcholine) and low-density lipoprotein (LDL) were from Sigma Chemical Co. (St. Louis, MO). According to HPLC analysis of phosphatidylcholine, it contained some α-, γ-, and δ-tocopherols (19, 117, and 59 μg/g, respectively). The fatty acid composition of phosphatidylcholine was similar to that of soybean oil. Ultraflo L, the enzyme preparation with β-glucanase as main activity and ferulic acid esterase as side activity, was provided by Novozymes (Bagsvaerd, Denmark).

Phenolic Standard Compounds. Sinapic acid, pyrogallol, catechin, ferulic acid, taxifolin, cyanidin-3-glucoside, and ellagic acid were from Extrasynthèse. Vinylsyringol, the main phenolic compound in postexpelled crude rapeseed oil, was synthesized according to Rein et al. (*23*).

Reference Materials. Five different types of hydrolyzable tannins were used as reference compounds. These tannins were chosen so that each of them represented different branches of the biosynthetic pathway of hydrolyzable tannins found in plants (*24*−*26*). Pentagalloylglucose (molar mass 940 g/mol); purity >99.9%) is the immediate biosynthetic precursor of both ellagitannins and gallotannins, and it was purified from commercially available tannic acid (Baker) as described by Salminen and Lempa (*27*). The gallotannin reference sample included octa- to tridecagalloylglucoses (molar masses 1396−2156 g/mol; purity >99.9%) and was isolated from tannic acid (Baker) by column chromatography with Sephadex LH-20 using the elution profile described in Salminen et al. (*28*). Pedunculagin (molar mass 784 g/mol; purity 95.8%) was used as a model compound for monomeric ellagitannins, and was isolated from leaves of *Betula nana* L. as described in Salminen et al. (*25, 28*). Oenothein B (molar mass 1568 g/mol; purity >99.9%) was the reference compound for dimeric ellagitannins consisting of monomers with two cyclic glucose (i.e., glucopyranose) units. It was isolated and purified from the flowering top of *Epilobium angustifolium* with a combination of Sephadex LH-20 and preparative HPLC as in Salminen et al. (*24, 27*). Cocciferin D_2 (molar mass 1868 g/mol; purity 94.3%) was isolated from leaves of *Quercus robur* as in Salminen et al. (*26*) and used as a model for dimeric ellagitannins consisting of monomers with both cyclic and acyclic glucose cores. Pinoresinol and matairesinol were isolated and purified from *Pinus sylvestris* bark as previously described in Karonen et al. (*8*).

Phenolic Extracts and Isolates. The rapeseed (*Brassica rapa* L.) meal (fat 9%, not removed) used was the residue of a rapeseed deoiling process, where the oil was expelled from the seeds at elevated temperature, and the crude postexpelled rapeseed oil was donated by Mildola Ltd, Finland. Enzymatic treatment of the rapeseed meal was

carried out according to Vuorela et al. (*7*) using Ultraflo L enzyme preparation. Phenolics were extracted with aqueous methanol (80%) from crude postexpelled rapeseed oil according to the method outlined by Koski et al. (*20*). Rapeseed phenolic extracts were used as solutions.

Fresh red raspberry (*Rubus idaeus* L, v. Ottawa) samples (5 kg) were purchased from a market during growing season of July−August 2002. Frozen raspberries were freeze-dried and extracted with aqueous acetone (70%) according to Kähkönen et al. (*29*). Raspberry anthocyanins and ellagitannins were isolated by using a method as described in Kähkönen et al. (*30*). For identification of ellagitannin monomers preparative HPLC followed by LC−ESI-MS analysis was used (*17*). The anthocyanin containing fraction was further purified by using preparative HPLC followed by analytical HPLC for compositional determination (*30*). Raspberry extracts and fractions were used as freeze-dried powders.

Scots pine (*Pinus sylvestris* L.) bark was collected in January 2001 in Honkajoki, Siikainen, Finland, air-dried, and ground by Ravintorengas Oy. Pine bark powder (150 g) was extracted with 70% aqueous acetone according to Karonen et al. (*8*). The water-soluble crude extract was first washed with *n*-hexane and then extracted with chloroform (pine bark fr. I). A part of the chloroform fraction was further fractionated by semipreparative HPLC (pine bark fr. II). Pine bark extracts and fractions were used as freeze-fried powders.

Phenolic Composition. The HPLC analyses were performed according to the method outlined by Koski et al. (*20*) for rapeseed, by Kähkönen et al. (*29*) for raspberries, and by Karonen et al. (*8*) for pine bark. The main phenolic composition of rapeseed, raspberry, and pine bark is shown in **Table 1**. The total phenolic content was determined in the solutions of rapeseed meal and oil phenolic extracts according to the method outlined by Vuorela et al. (*7*). Sinapic acid was used as a standard compound.

Antioxidant Activity Testing. For antioxidant testing, the dry extracts were dissolved in methanol. For DPPH free radical scavenging assay, the rapeseed meal extract was dried and then dissolved in methanol; for liposome and for LDL model systems, the extract was in the extraction solvent.

The DPPH radical scavenging test was performed by the method outlined by Kähkönen et al. (*31*). The methanolic extracts and standards were tested in triplicate at concentrations of 0.5 and 1.0 mg/mL. Pyrogallol (0.5 mg/mL) was a control sample in each measurement with activities between 90% and 96%. The results were expressed as the percentage of radicals scavenged after 4 min of reaction time.

Liposome Model System. The liposomes were prepared from soybean lecithin, and the concentration of phosphatidylcholine in samples was 0.8 mg/mL. The concentrations of the extracts and reference materials were 4.2 and 8.4 μg/mL calculated as total phenolics. The concentrations of the standards were 10 and 25 μM. The analyses were performed in triplicate. The inhibition against liposome oxidation was calculated at day 3 by measuring the formation of hexanal by headspace gas chromatography according to the method of Huang and Frankel (*32*).

LDL Oxidation. The LDL oxidation was performed as described by Kähkönen et al. (*31*) using human LDL, which was diluted to a protein concentration of 0.2 mg/mL using phosphate buffer. The concentrations of the tested extracts were 1.4 and 4.2 μg/mL calculated as total phenolics. The standards were tested at concentrations of 10 and 25 μM.

Antiinflammatory Testing. The antiinflammatory properties of the extracts and standards were tested by measuring their effects on the proinflammatory mediators nitric oxide (NO) and prostaglandin E_2 (PGE_2) in activated macrophages. The extracts were tested at concentrations of 20 and 50 μg/mL except rapeseed meal extract in dilutions of 1:500 and 1:100 and rapeseed oil in dilutions of 1:1000 and 1:500.

Cell Culture. J774 murine macrophages (American Type Culture Collection, Rockville, MD) were cultured at 37 °C (in 5% carbon dioxide) in Dulbecco's modified Eagle's medium with glutamax-I. Culture media contained 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (all from Gibco, Paisley, Scotland). The cells were harvested with trypsin-EDTA. The cells were seeded on 96-well plates for the XTT test, and on 24-well plates for NO and PGE_2 measurements. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium. The tested compounds were first dissolved in DMSO, and the stock solution was diluted to 1:1000 in the culture medium to obtain the final concentrations. NO and PGE_2 production was induced by LPS (100 ng/mL).

XTT Test. Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN). The cells were incubated with the tested compounds for 20 h before the addition of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) (final concentration 0.3 mg/mL) and *N*-methyldibenzopyrazine methyl sulfate (1.25 mM). The cells were then further incubated for 4 h, and the amount of formazan accumulated in the growth medium was assessed spectrophotometrically. Triton-X-treated cells were used as a positive control. The conditions were considered toxic if the cells' ability to metabolize XTT to formazan was lowered by more than 20% in comparison to that of the untreated cell culture.

Nitric Determination. Measurement of nitrite accumulation in the culture medium was used to determine NO production. The culture medium was collected after 24 h incubation, and the nitrite concentration was measured by Griess reaction.

Prostaglandin E_2 Assays. The culture medium was collected after 24 h incubation, and PGE_2 concentrations were determined by radioimmunoassay using reagents from the Institute of Isotopes (Budapest, Hungary).

Mutagenicity Testing. The mutagenic effects were determined according to Flamand et al. (*33*) using *Salmonella typhimurium* strains TA98 and TA100 (Wenometrix Inc., San Diego, CA) grown in nutrient broth (Beckton Dickinson, le Pont de Claix, France) at 37 °C grown for 24 h. The test was performed with buffer (B) or in the presence of 10% rat liver enzyme S9-mix (S9) as a metabolic activator. 2-Nitrofluorone (2N) 0.1 μg/well was used as a positive control when B was present, for TA98, and 0.05 μg/well of sodium azide (Na) for TA100. When S9 was present, 2-aminoanthracene (2A) was used as a positive control, for TA98 strain 0.5 μg/well and for TA100 0.75 μg/well, respectively. The final dilutions in wells were 1/27 of the original extract concentrations. The results were expressed as positive (+) or negative (−) effects comparing to the control sample.

The antimutagenic properties were determined according to Yen et al. (*34*) using TA98 and TA100 strains with buffer (B) and with (S9) metabolic activation. The vinylsyringol or the extracts together with positive controls (see above) were added in wells, and they were incubated for 48 h.

Antimicrobial Testing. The antimicrobial activity of the rapeseed, raspberry, and pine bark extracts and standards was tested using following bacterial strains colonizing in the colon: *Escherichia coli* (FOMK), *Salmonella typhimurium* (TA100), *Klebsiella oxytoca* (FOMK), *Proteus mirabilis* (FOMK), *Lactobacillus acidophilus* (ATCC 4356), and *Lactobacillus crispatus* (A269-21). Strains were grown in nutrient broth (Becton Dickinson, Le Pont de Claix, France) at 37 °C before the test. The antimicrobial assay was performed according to Skyttä and Mattila-Sandholm (*35*) in 96-well plates at concentrations of 4−100 μg/mL. The samples were incubated for 24 h, and the test was performed in triplicate.

Drug Permeability Testing. In this test, the aim was to see if the rapeseed and pine bark extracts or fractions affect the absorption of the coadministered widely used highly permeable drugs verapamil, metoprolol, paracetamol, and ketoprofen. The test was performed according to the method outlined by Laitinen et al. (*35*). In short, the Caco-2 cells (American Type Culture Collection, Rockville, MD) were seeded at 6.8×10^4 cells/cm^2 onto polycarbonate filter membranes in clusters of 12 wells (Corning Coster Corp., Cambridge, MA). The cells were grown in a medium consisting of DMEM containing 4.5 g/L glucose supplemented with 10% FBS, 1% NEAA, 1% L-glutamine, penicillin (100 IU/mL), and streptomycin (100 μg/mL). The cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air, at 95% relative humidity. The growth medium was changed three times a week. Cells from passage numbers 31 to 42 were used for the transport experiments. The cell monolayers were used in experiments at ages ranging from 21 to 28 days. The apical solution was changed to HBSS containing the drugs and/or the extracts. The permeabilities of vera-

Table 2. Radical Scavenging (DPPH Test) and Antioxidant Activity of Rapeseed, Raspberry and Pine Bark Phenolics, Phenolic Reference Materials, and Standards (Percent Inhibition ± SD)

	DPPH[b]		liposome[b]	
extracts[a]	0.5 mg/mL	1.0 mg/mL	4.2 μg/mL	8.4 μg/mL
rapeseed meal ex	38.5 ± 0.1 c	46.3 ± 0.2 c	96.9 ± 0.8 a	98.7 ± 0.1 a
rapeseed oil ex	65.4 ± 4.7 a	93.8 ± 0.9 a	98.2 ± 0.1 a	98.2 ± 0.1 a
raspberry ex	46.7 ± 1.6 b	55.7 ± 1.6 c	53.5 ± 2.4 c	72.0 ± 2.0 d
raspberry et	65.8 ± 0.9 a	87.7 ± 3.9 b	57.3 ± 4.1 c	57.3 ± 1.4 e
raspberry as	28.7 ± 1.0 c	47.6 ± 1.9 d	81.8 ± 5.4 b	91.9± 1.0 b
pine bark ex	16.4 ± 1.4 d	32.8 ± 1.1 e	64.3± 1.1 c	34.9 ± 0.1 f
pine bark fr. I	8.9 ± 2.1 e	12.5 ± 2.3 f	80.5 ± 1.6 b	91.8 ± 0.8 b
pine bark fr. II	5.3 ± 1.2 f	9.6 ± 1.1 f	64.4 ± 1.1 b	89.4 ± 0.3 c
reference mat.	0.5 mg/mL	1.0 mg/mL	4.2 μg/mL	8.4 μg/mL
pedungulagin	85.8 ± 1.0	94.2 ± 1.9	73.9 ± 9.9	75.6 ± 5.2
pentagalloylglucose	95.6 ± 0.2	96.1 ± 0.6	76.1 ± 0.3	87.4 ± 2.8
gallotannins	96.0 ± 0.3	95.5 ± 0.4	83.1 ± 3.5	93.4 ± 2.1
cocciferin D_2	91.3 ± 1.3	95.2 ± 1.6	27.8 ± 19.6	12.7 ± 5.1
oenothein B	82.6 ± 1.7	95.3 ± 0.3	14.1 ± 5.6	22.4 ± 4.9
pinoresinol	18.8 ± 1.3	28.8 ± 2.3	94.1 ± 0.6	97.1 ± 0.0
matairesinol	10.7 ± 0.3	16.7 ± 1.9	89.4 ± 1.5	95.5 ± 0.7
standards	0.5 mg/mL	1.0 mg/mL	10 μM	25 μM
sinapic acid	47.6 ± 2.8	90.8 ± 0.7	96.7 ± 0.8	96.6 ± 0.3
ferulic acid	34.7 ± 2.2	43.2 ± 2.8	90.2 ± 0.3	97.0 ± 0.2
catechin	93.0 ± 2.2	94.6 ± 0.5	86.2 ± 0.8	96.4 ± 2.6
ellagic acid	87.3 ± 1.6	90.4 ± 1.6	98.3 ± 0.3	98.6 ± 0.3
taxifolin	55.1 ± 5.7	85.5 ± 1.6	79.2 ± 2.8	92.8 ± 0.1
cya-3-glu	35.2 ± 0.9	65.2 ± 1.1	89.4 ± 0.2	97.1 ± 0.1
vinylsyringol	37.1 ± 3.7	78.7 ± 3.3	93.0 ± 5.0	97.7 ± 0.9

[a] Phenolic extract of rapeseed meal (rapeseed meal ex), phenolic extract of crude rapeseed oil (rapeseed oil ex), raspberry phenolic extract (raspberry ex), raspberry ellagitannins fraction (raspberry et), raspberry anthocyanin fraction (raspberry as), aqueous acetone (70%), extract of pine bark (pine bark ex), chloroform fraction of pine bark phenolic extract (pine bark fr. I), and chloroform subfraction of pine bark phenolic extract (pine bark fr. II). [b] Values in the same column with different letters are significantly different ($p < 0.05$).

pamil, metoprolol, paracetamol, and ketoprofen across Caco-2 cell monolayers were studied in an apical-to-basolateral direction at pH 7.40. Samples were obtained after 15, 30, 45, 60, and 90 min. Drug concentrations were determined using HPLC. Monolayer integrity was determined by measuring transepithelial electrical resistance (TEER) and by using ^{14}[C]-mannitol. Apparent permeability coefficients, P_{app} (cm/s), were calculated using the equation $P_{app} = \Delta Q/(\Delta t A C_o)$, where $\Delta Q/\Delta t$ is the flux of compound across the monolayers, A (cm^2) is the surface area of the cell monolayer, and C_0 is the initial concentration of the compound in the donor (apical) compartment. Results reported are average P_{app} (cm/s) values ± SD ($n = 3$). The P_{app} values relating to drugs with extracts were compared to P_{app} values relating to drugs without extracts (controls). Percentage differences in the P_{app} values from control P_{app} values were calculated.

MTT Test. This colorimetric assay was used to determine cell viability (mitochondrial activity) by measuring the extent of formazan formation after solubilization of the Caco-2 cells. Briefly, cells were seeded onto 96-well plates (Costar Corp., Cambridge, MA) at a density of 5.0×10^4 cells/well and incubated for 20−24 h. The cells were exposed for 60 min to solutions of the drugs, extracts, or drugs and extracts, at 37 °C. Subsequently, the medium was aspirated, MTT solution (5 mg/mL) was added, and the cells were further incubated for 1.5 h. Formazan crystals were then dissolved in a solution of 10% SDS and 0.01 M HCl in isobutanol. The color developed was measured at 590 nm. Results ($n = 8$) were expressed as percentages of the control value (cells treated with NBSS only), and lowering over 20% was evaluated as toxic conditions.

Statistical analysis was performed using Statgraphics (STCC Inc., Rockville, ML) one-way ANOVA. The permeability results were confirmed statistically by unpaired t test combined with Dunn−Sidak adjusted probability and bonferroni adjusted probability tests using SYSTAT version 10.2 for Windows (SYSTAT Software Inc., Richmond, CA).

RESULTS

Antioxidant Activity. The antioxidant activity of the extracts was tested in DPPH free radical scavenging test and in the liposome and LDL model systems. Crude rapeseed oil phenolics and raspberry ellagitannins were excellent free radical scavengers followed by raspberry phenolic extract and raspberry anthocyanins. Pine bark phenolics as well as rapeseed meal phenolic extract were not potent free radical scavengers as their activities were under 50% at a concentration of 1.0 mg/mL. All the tannin reference materials exhibited radical scavenging activities of over 90% at a concentration of 1.0 mg/mL while the lignans, pinoresinol and matairesinol, showed only weak radical scavenging activity. Ellagic acid, catechin, and sinapic acid were the most effective standard compounds as radical scavengers. In the liposome model system, phenolic extracts of rapeseed oil and meal were excellent inhibitors of hexanal followed by raspberry anthocyanins and pine bark fractions at a concentration of 8.4 μg/mL. Of the tested reference materials, pinoresinol and matairesinol were the most effective antioxidants followed by gallotannins and pentagalloylglucose toward oxidation of liposomes. All the tested standards investigated exhibited excellent or good antioxidant activity in inhibiting the formation of hexanal in liposomes (**Table 2**). Apart from ferulic acid, the standards tested as well as raspberry ellagitannins, raspberry phenolic extract (only at higher concentration), and pine bark semipreparative fraction were excellent antioxidants toward oxidation of LDL (**Table 3**).

Antiinflammatory Properties. To rule out possible cytotoxic effects, extracts and standards were first tested using the XTT test to measure cell viability. Catechin (100 μM) was toxic to the cells in the incubation conditions used, and therefore it was excluded from further antiinflammatory tests.

The effects of the rapeseed and raspberry phenolics on the formation of proinflammatory mediators NO and PGE_2 were measured. Crude rapeseed oil phenolic extract and raspberry anthocyanins had antiinflammatory properties (**Table 4**). At a

Table 3. Antioxidant Activity of Some Extracts and Standards in the LDL Model System

	LDL	
extracts[a]	1.4 μg/mL	4.2 μg/mL
raspberry extract	–14.9 ± 1.1	96.7 ± 1.2
raspberry ellagitannins	94.8 ± 0.5	97.3 ± 0.1
pine bark fr I	10.2 ± 7.4	16.9 ± 3.5
pine bark fr II	nd[b]	86.3 ± 1.3
standards	10 μM	25 μM
sinapic acid	28.0 ± 11.9	95.3 ± 0.1
ferulic acid	11.1 ± 1.4	38.6 ± 2.4
catechin	97.7 ± 1.3	98.6 ± 0.2
ellagic acid	11.1 ± 2.6	98.6 ± 0.0
taxifolin	87.6 ± 0.1	94.9 ± 0.3
cya-3-glu	82.4 ± 0.9	91.0 ± 1.0
vinylsyringol	7.5 ± 0.7	97.1 ± 0.1

[a] Raspberry and pine bark extracts as described in **Table 2**. [b] Not determined.

concentration of 1:500, rapeseed oil phenolics inhibited PGE_2 formation by 54%, and raspberry extract, raspberry ellagitannins, and raspberry anthocyanins (all in 50 μg/mL) suppressed PGE_2 by 40%, 35%, and 71%, respectively. Rapeseed oil phenolics (1:500) reduced NO production slightly (17% inhibition). Vinylsyringol was the most effective standard compound against formation of PGE_2 as it reduced PGE_2 formation over 96% also at the lower concentration (20 μg/mL). Sinapic acid, taxifolin, and pinoresinol (10−100 μM) reduced PGE_2 production in a dose dependent manner. In addition, matairesinol (100 μM) inhibited formation by 70% while cyanidin-3-glucoside had no effect. Rapeseed meal extract obtained by enzymatic treatment had no antiinflammatory properties. Although the antiinflammatory properties of pine bark have previously been published by Karonen et al. (*8*), the present study showed that the phenolic compounds found in pine bark, taxifolin, matairesinol, and pinoresinol reduce NO production in a dose dependent manner.

Mutagenicity Test. Mutagenic properties of the rapeseed and pine bark extracts and vinylsyringol were tested. It was shown that none of them had mutagenic properties when *S. typhimurium* strains TA98 and TA100 were used with buffer (B) and with metabolic activation (S9). The antimutagenic test (**Table 5**) showed a slight decrease in mutagenicity of 2-nitrofluorone (2N) for both used strains for all samples except pine bark fr. I, which showed an increase in the amount of colonies of TA100. In the presence of the metabolic activator (S9) and 2-aminoanthracene (2A) the effect was slightly increasing in most of the cases, except again for pine bark fr. I, which showed an increase in the formation of bacterial colonies with 2A and TA100. The in vitro mutagenic potential of raspberry phenolics is reported elsewhere (*22*).

Antimicrobial Activity. The antimicrobial activity of the extracts against some human colonizing bacteria was assayed. It was shown that raspberry had the most powerful antimicrobial activity. Raspberry extract and raspberry ellagitannin fraction inhibited the growth of *K. oxytoca* and *P. mirabilis* and had a minor inhibitory effect on the growth of *S. typhimurium* (**Table 6**). Raspberry anthocyanins inhibited the growth of *L. acidophilus*. Rapeseed and pine bark extracts had only minor effects on the growth of the tested microorganisms (**Table 6**).

Cytotoxicity Tests. Extracts and fractions were not cytotoxic to macrophages or Caco-2 cells in the test systems applied.

Effects on Cell Permeability. The effect of the rapeseed and pine bark phenolics as well as vinylsyringol on verapamil, ketoprofen, metoprolol, and paracetamol permeability was investigated (**Figure 1**). Rapeseed oil phenolics had no effects on the permeability of the model drugs (**Figure 1D**) while rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen (**Figure 1E**). The permeability of metoprolol was enhanced by vinylsyringol and pine bark phenolics.

DISCUSSION

Rapeseed. *Antioxidant Activity*. Rapeseed oil phenolics were excellent radical scavengers. In earlier studies (*20, 21*), it was concluded that vinylsyringol, the main phenolic compound in postexpelled crude rapeseed oil, is principally responsible for the radical scavenging activity of the oil. Also Kuwahara et al. (*36*) found that rapeseed oil canolol, a compound identical to vinylsyringol, had effective antiradical capacity against the endogenous mutagen peroxynitrite. In the present study, the radical scavenging activity of sinapic acid was higher than that of vinylsyringol. However, rapeseed oil phenolics consisting mainly of vinylsyringol (87%) with minor amounts of sinapic acid and its conjugate (13%) was more active compared to vinylsyringol and sinapic acid alone. Thus, there may be synergistic effects between the different rapeseed phenolics. In rapeseed meal, the radical scavenging activity of the phenolics was only moderate, which finding is in accordance with our previous results (*7*). Amarowicz et al. (*36*) found that the radical scavenging activity of phenolic compounds isolated from

Table 4. Antiinflammatory Effect of Rapeseed and Raspberry Phenolics and Phenolic Standard Compounds

	NO production inhibition (%)		PGE_2 inhibition (%)	
extracts[a]	20 μg/mL	50 μg/mL	20μg/mL	50 μg/mL
rapeseed meal extract	6.9 ± 1.7[b]	12.2 ± 2.1[b]	–96.9 ± 16.4[b]	–62.0 ± 11.7[b]
rapeseed oil extract	9.8 ± 1.0[c]	17.4 ± 1.9[c]	17.9 ± 4.2[c]	54.0 ± 3.2[c]
raspberry extract	–2.6 ± 1.9	5.7 ±	34.6 ± 8.0	39.6 ± 5.3
raspberry ellagitannins	17.1± 3.3	4.8 ± 3.7	34.6 ± 1.8	34.6 ± 10.1
raspberry anthocyanins	–10.7± 2.7	–4.2 ± 1.3	50.4 ± 9.2	70.9 ± 2.5
standards	10 μM	100 μM	10 μM	100 μM
sinapic acid	8.9 ± 1.9	5.2 ± 1.3	13.0 ± 7.8	58.2 ± 3.7
vinylsyringol	28.5 ± 1.8[d]	43.8 ± 0.7[d]	96.6 ± 0.4[d]	96.7 ± 0.4[d]
catechin	5.9 ± 1.9	tox.		tox.
taxifolin	3.1 ± 1.8	23.9 ± 1.8	34.8 ± 3.7	82.5 ± 0.6
pinoresinol	5.9 ± 1.2	52.5 ± 1.1	45.3 ± 2.1	88.4 ± 1.7
matairesinol	–5.2 ± 0.9	23.1 ± 1.0	–2.2 ± 6.3	70.3 ± 1.9
cya-3-glc	–4.9 ± 3.8	5.3 ± 1.8	16.3 ± 2.9	5.4 ± 5.5
ellagic acid	–15.0 ± 3.0	38.2 ± 1.9	–114.6 ± 20.7	–291.6 ± 11.1

[a] Rapeseed and raspberry and extracts and isolates as described in **Table 2**. [b] Tested in dilutions of 1:500 and 1:100. [c] Tested in dilutions of 1:1000 and 1:500. [d] Tested at concentrations of 20 and 50 μg/mL.

Table 5. Antimutagenicity Activity of Rapeseed and Pine Bark Phenolics and Vinylsyringol Showing the Amount of Colonies Formed in Comparison to B or S9 Control

extracts[a]	TA98 + 2N + B[b]	TA98 + 2A + S9	TA100 + Na + B	TA100 + 2A + S9
rapeseed meal extract	3.3	6.1	2.6	6.0
rapeseed oil extract	4.0	6.0	2.2	4.4
vinylsyringol	4.8	6.9	2.8	4.6
pine bark ex	3.7	6.4	2.2	5.1
pine bark fr. I	3.0	6.9	5.4	10.1
pine bark fr. II	3.5	6.8	2.0	5.2
na[c]	5.6	5.2	3.8	4.8

[a] Rapeseed, raspberry, and pine bark extracts as described in **Table 2**. [b] *Salmonella typhimurium* strains TA98 and TA100 with buffer (B), with metabolic activation (S9), 2-nitrofluorone (2N), 2-aminoanthracene (2A), buffer (B), sodium azide (Na). [c] Nothing added, only positive control.

rapeseed hulls varied 6−81% (1 mg/mL) depending on fraction. None of these fractions contained free sinapic acid.

Rapeseed phenolics exhibited excellent antioxidant activity toward oxidation of liposomes. In rapeseed oil, the antioxidant activity is most likely due to vinylsyringol. Also rapeseed meal phenolics extracted with the aid of Ultraflo L enzyme preparation showed excellent antioxidant activity, but this effect is mainly due to sinapic acid. These findings are in accordance with our previous studies (*7, 20*). The antioxidant activity of hydroxycinnamic acids has been investigated earlier. Andreasen et al. (*38*) found caffeic acid with two hydroxyl groups more effective than sinapic acid in the LDL model system, but sinapic acid was more effective than ferulic acid or *p*-coumaric acid. According to Pekkarinen et al. (*39*), the radical scavenging activity was higher in hydroxycinnamic acids with two hydroxyl groups (caffeic acid) due to electron-donating ability. In addition to one hydroxyl group, a second methoxy group in sinapic acid increased the radical scavenging activity more than hydrogen in ferulic acid.

Antiinflammatory Properties. Crude rapeseed oil phenolics inhibited the formation of NO and PGE_2; therefore they exhibited antiinflammatory properties. On the other hand, rapeseed meal phenolics extracted with ferulic acid esterase showed no antiinflammatory properties. It is concluded that the antiinflammatory properties of rapeseed oil phenolics are due to vinylsyringol, which effectively inhibited the formation of NO and PGE_2. The antiinflammatory effects of sinapic acid were weaker, which partly explains the lack of effect with rapeseed meal phenolics consisting mainly of sinapic acid (64%).

Antimutagenic Properties. Rapeseed extracts and vinylsyringol had no antimutagenic properties in the modified Ames test with S9 mix. However, without S9 mix, all tested samples showed antimutagenic properties, which means that all tested samples were antimutagenic to prokaryotic cells. This finding is in accordance with Kuwahara et al. (*37*), who concluded that canolol is an antimutagenic compound without S9 mix. The antimutagenic potency of canolol was reported higher than that of some flavonoids as well as α-tocopherol.

Effects on Drug Permeability. Rapeseed oil phenolic extract had no significant effects on the permeability of the model drugs, whereas pure vinylsyringol enhanced the permeability of metoprolol, a model drug passively permeating the cell membrane. Rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen, indicating that sinapic acid may have an impact on drugs and other components being actively transported across the cell membrane.

Raspberry. *Antioxidant Activity*. Raspberry phenolic extract with 51% ellagitannin content and the 95% pure raspberry ellagitannin fraction exhibited good radical scavenging activity, which is in accordance with the radical scavenging activity of the tannin reference materials tested. Pedunculagin, pentagalloyl glucose and gallotannins were antioxidants also toward liposome oxidation. On the other hand, cocciferin D_2 and oenothein B are dimeric ellagitannins, and it seems that their structures were not ideal for incorporation into the liposome membrane and the subsequent inhibition of lipid oxidation. The hydrolysis product of ellagitannins, ellagic acid, showed good radical scavenging activity, and excellent antioxidant activity toward both liposome and LDL oxidation. These results are in accordance with Kähkönen et al. (*17*) showing that ellagitannins isolated from red raspberries (*Rubus idaeus* L.) are effective as DPPH radical scavengers and excellent antioxidants toward oxidation of LDL and methyl linoleate emulsion. Sanquiin H-6 (dimer) and to a minor extent lambertianin C (trimer), the main ellagitannins in raspberries, were reported to exhibit antioxidant activity (*16, 17*).

Also raspberry anthocyanins showed good antioxidant activity toward oxidation of liposomes. In the LDL model system, raspberry phenolic extract and raspberry ellagitannins showed excellent antioxidant activity. For effective antioxidant activity it is important that the antioxidants locate near the membrane surface in order to act as antioxidants. According to Castelli et al. (*40*) liposomes are a suitable model for studying the membrane structure and properties due to their structural similarity to the lipid matrix of cell membranes. Raspberry antioxidant activity has been investigated earlier in several antioxidant tests (*12, 14, 15, 17, 29, 30, 41*). Viljanen et al. (*12*) found raspberry anthocyanins to be the most active berry phenolics followed by raspberry phenolic extract and raspberry ellagitannins in inhibiting the oxidation of a liposome−protein model system. This finding is in accordance with our present results of liposome oxidation.

Antiinflammatory Properties. Raspberry phenolics, especially anthocyanins and ellagitannins, affected the formation of nitric oxide and PGE_2, which are proinflammatory mediators. The activity of the standard compounds tested, ellagic acid and cyanidin-3- glucoside, was only weak or negative. Hämäläinen

Table 6. Antimicrobial Activity of the Rapeseed, Raspberry, and Pine Bark Phenolics (Inhibition % ± SD)

extracts[a]	*K. oxytoca*	*S. typhimurium*	*L. acidophilus*	*L. crispatus*	*E. coli*	*P. mirabilis*
rapeseed meal ex	nd	13.5 ± 0.4	nd	nd	nd	nd
rapeseed oil ex	nd	15.4 ± 2.8	nd	nd	nd	nd
raspberry ex	33.7 ± 1.3	10.5 ± 0.7	nd[b]	nd	nd	62.3 ± 1.4
raspberry et	31.2 ± 1.0	13.5 ± 1.3	nd	nd	4.8 ± 0.7	59.1 ± 0.9
raspberry as	nd	nd	40.4 ± 1.3	nd	nd	nd
pine bark ex	nd	12.1 ± 1.4	nd	nd	nd	8.9 ± 1.8
pine bark fr. I	nd	18.0 ± 2.6	nd	nd	nd	nd
pine bark fr. II	nd	nd	nd	nd	nd	nd

[a] Extracts as described in **Table 2**. [b] Not detected.

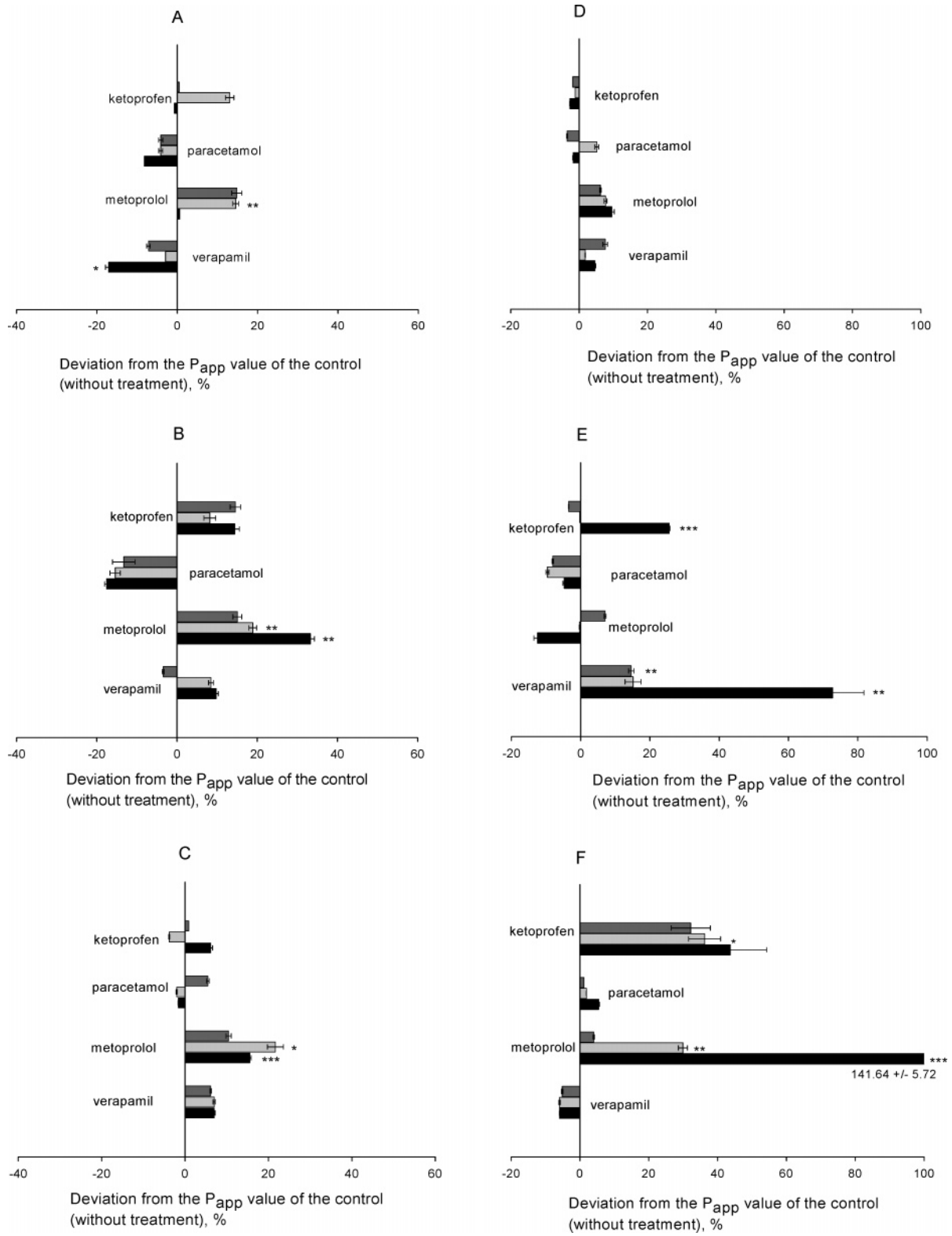

Figure 1. Changes in permeabilities of verapamil, metoprolol, paracetamol, and ketoprofen in the presence of various concentrations of pine bark extract (**A**), pine bark fr. I (**B**) (1.0; 0.1; 0.01 mg/mL), and pine bark fr. II (**C**) (0.1; 0.01; 0.001 mg/mL); rapeseed oil extract (**D**), meal extract (**E**), and vinylsyringol (**F**) (1.0; 0.1; 0.01 mg/mL) across Caco-2 cell monolayers. Extracts and fractions of rapeseed and pine bark are as described in **Table 2**. Values are mean percentage differences ($n = 3$) ± SDs from the permeabilities of the compounds across Caco-2 cell monolayers in the absence of the extracts (control values) ± SD. Black staples represent the strongest, light gray staples the medium, and dark gray staples the mildest concentration of the studied extracts. *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$.

et al. (*42*) found some flavonoids effective as antiinflammatory compounds as they inhibited iNOS protein and mRNA expression and nitric oxide formation. Pelargonidin, an anthocyanidin compound, was one of the most effective phenolics.

Antimicrobial Activity. Raspberry phenolic extract and its ellagitannin fraction had powerful antimicrobial properties against the growth of *K. oxytoca* and *P. mirabilis*. These bacteria are human colonial pathogens. Raspberry anthocyanins had no

effects against these bacteria. According to Puupponen-Pimiä (*43*), the excellent antimicrobial activity of berries, especially the genus of *Rubus*, may be due to the complex phenolic polymers such as ellagitannins, and the degree of hydroxylation affects the antimicrobial activity of pure phenolic compounds. Raspberry phenolics were mainly reported to inhibit the growth of Gram-negative bacteria such as *Staphylococcus* and *Salmonella* but with no effect on Gram-positive lactic acid bacteria (*43, 44)*. However, in our present study, raspberry anthocyanins were found to exhibit strong inhibiting effects on the growth of *L. acidophilus*, a Gram-positive bacterium. This finding may be of importance when consuming raspberry anthocyanins in high concentrations because *L. acidophilus* is commonly used in fermented milk products. In addition, Rauha et al. (*6*) found raspberry also to strongly inhibit the growth of *Bacillus subtilis* and *Micrococcus luteus*.

Pine Bark. *Antioxidant Activity*. Pine bark chloroform fraction and semipreparative fraction showed good antioxidant activities against oxidation of liposomes and LDL particles. In these fractions, ferulic acid as well as the lignans pinoresinol and matairesinol are the main phenolic compounds. Further characterization revealed that the semipreparative fraction contained low-molecular-weight phenolics and lignan type compounds (*45*) in addition to a novel dihydroflavonol (*46*). In these fractions, the main phenolic constituents, ferulic acid as well as pinoresinol explain the good antioxidant activity of pine bark phenolic fractions. The oxidation mechanism of lignans is not radical scavenging, but some other mechanism, for example metal chelation. This finding is in accordance with a previous study of Morelló et al. (*47*). Kähkönen et al. (*5*) tested the antioxidant activity of pine bark phenolics in methyl linoleate model system and found pine bark an excellent antioxidant at 5000 ppm concentration. Grimm et al. (*48*) found that pycnogenol metabolites from maritime pine bark extract were effective superoxide radical scavengers.

Antiinflammatory Activity. Earlier it was reported by Cho et al. (*49*) that pycnogenol, which is a phenolic extract from maritime pine (*Pinus maritima)* bark including phenolic compounds such as catechin, epicatechin, taxifolin, and proanthocyanidins (*50*), can inhibit the proinflammatory cytokine interleukin-1 production. Karonen et al. (*8*) reported antiinflammatory properties of pine bark (*Pinus sylvestris* L.) phenolics, especially ferulic acid, pinoresinol and matairesinol being effective reducers of NO and PGE_2 production. In the present study, the antiinflammatory effect of pinoresinol and matairesinol, which are the major compounds in pine bark semipreparative fraction, was investigated. It was shown that pinoresinol and matairesinol inhibited the formation of nitric oxide and prostaglandin E_2. When comparing the antiinflammatory activity of the phenolic standards, pinoresinol explains most of the antiinflammatory activity of pine bark phenolics.

Effects on Drug Permeability. The permeability of metoprolol was affected by all pine bark samples, whereas they had no significant effects on the permeability of the other model drugs. Here the effect was enhancing, whereas Laitinen et al. (*9*) reported that scots pine bark extract adversely affected the transport of verapamil and metoprolol. This can be explained by the final content of the different extracts and fractions, although originating from similar material. Tammela et al. (*51*) found that the permeabilility of pure flavonoids depends on the degree of hydroxylation and molecular configuration, but in contrast to other flavonoids catechin and epicatechin did not penetrate the cell membrane in the Caco-2 cell model. Further, our study showed only minor effects on permeability of the acidic drug compounds, which is in accordance with Laitinen et al. (*9*).

In conclusion, raspberry ellagitannins exhibited good radical scavenging activity and were effective toward LDL oxidation, although partly due to structural hindrance they did not inhibit liposome oxidation in contrast to raspberry anthocyanins. Raspberry ellagitannins were effective in inhibing the growth of human colonizing microorganisms, *P. mirabilis* and *K. oxytoca*, while raspberry anthocyanins inhibited the growth of *L. acidophilus*. Both anthocyanins and ellagitannins in raspberry contributed to the antiinflammatory effect of raspberry phenolics. Vinylsyringol present in postexpelled crude rapeseed oil and sinapic acid in the rapeseed oil pressing meal residue exhibited excellent antioxidant properties. The antiinflammatory properties of crude rapeseed oil are due to vinylsyringol, which effectively inhibited the formation of NO and PGE_2. Vinylsyringol also affected the cell permeability tested with a passively transported drug, metoprolol, while sinapic acid may have an impact on drugs and other components being actively transported across the cell membrane. Pine bark phenolics, especially the lignans, matairesinol and pinoresinol, were effective against formation of prostaglandin E_2, which is a proinflammatory mediator. The pine bark lignans did not exhibit radical scavenging properties, but inhibited the oxidation of liposomes most likely due to chelation. The permeability of metoprolol was affected by pine bark phenolics. None of the tested extracts were mutagenic nor cytotoxic to Caco-2 cells or macrophages. Thus, phenolic isolates from raspberry, pine bark, and rapeseed are bioactive and safe for possible food applications including functional foods intended for health benefit.

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