Effects of Extracts of Commonly Consumed Food Supplements and Food Fractions on the Permeability of Drugs Across Caco-2 Cell Monolayers

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Purpose. Extracts made from berries, herbs, and various plant materials, which might possess a range of activities, are used as health promoting products. Because little is known about their effects on the absorption of co-administered drugs, the effects of some food supplements, Finnish berries, and herbs were studied on the permeability of some commonly used drugs.

Methods. The permeabilities of verapamil, metoprolol, ketoprofen, paracetamol, and furosemide were studied across Caco-2 cell monolayers with contemporaneously administered extracts from flax seed, purple loosestrife, and Scots pine bark; bilberries, cowberries, and raspberries; oregano, rosemary, and sage. Toxicological tests were conducted to determine cellular damage.

Results. The effects of extracts on drug permeabilities were generally minor. Flax seed decreased the permeability of all drugs except verapamil. Purple loosestrife and pine decreased verapamil and metoprolol permeability. Changes caused by berries were mainly pH-related. Rosemary and oregano enhanced furosemide permeability.

Conclusions. Ingestion of extracts of herbs and berries studied are not expected to markedly change the permeabilities of highly permeable drugs. Harmful effects at sites of or during absorption are unlikely. However, if high doses of extracts are administered with low permeable drugs *in vitro,* effects on drug permeabilities could not be excluded. Use of such extracts should therefore be evaluated during continuous medication.

KEY WORDS: berries; Caco-2 permeability; food-drug interactions; food supplements herbs.

INTRODUCTION

The efficacies of orally administered drugs can be affected by food. The human diet contains macro- and micronutrients, non-nutrients, and additives. Studies to determine the effects of macronutrients (carbohydrate, fat, protein) on drug absorption and metabolism have been undertaken in healthy subjects under controlled conditions because diet varies between individuals (1). Changes in micro- and macronutrient compositions of the diet can increase absorption and/or elimination of drugs (2–5).

Because interest in preventive health care has increased, the food industry is producing products fortified not only with calcium, iron, magnesium, aluminum and vitamins, but also with various fractions of extracts of herbs, berries and other plant materials known to have beneficial activities, including antioxidative and antimicrobial. Such fortification could result in interactions if drugs were taken contemporaneously. Some drugs appear to be safe when taken with food but less so if taken with fortified foods (6). Mechanisms of interactions between drugs and fortified foods are similar to well known mechanisms of interactions between drugs and minerals (chelation, adsorption) and between drugs and secondary metabolites (e.g., the flavonoid naringenin, an inhibitor of the cytochrome P 450 enzyme family, is present in grapefruit juice). Absorption of some drugs may be decreased or excretion increased as a result of changes in gastric and/or urinary pH levels. Results of such interactions may be clinically insignificant but can be severe, resulting in treatment failure, need for frequent dose changes, development of antibiotic resistance, and increases in morbidity and mortality (6).

It has been reported that 31% of patients who consume herbal supplements are also taking prescribed drugs, and that about two thirds of such patients consume the herbal supplements without reporting this to those responsible for care of their health (7). The U.S. Food and Drug Administration (FDA) has also been reported to have received more than 2600 reports of serious problems involving herbal food supplements between 1993 and 1998, including 184 deaths. It is therefore important to explore possible interactions between food supplements and widely used drugs.

Ingestion of many herbal food supplements results in interactions with drugs taken at the same time. Many herbal food supplements contain large amounts of flavonoids. These can interact with efflux proteins present in absorptive enterocytes, and there may be severe interactions with drugs administered contemporaneously (8–10). Also consumption of bilberries (*Vaccinum myrtillus* L.) and raspberries (*Rubus idaeus* L.), which is widespread in Finland, at the same time as a nonsteroidal anti-inflammatory (e.g., acetylsalicylic acid or ketoprofen) can result in hemorrhage, as a result of additive antiplatelet effects (7).

Caco-2 cell cultures are widely used as an *in vitro* model in drug-absorption studies. The model is useful in determining roles played by various physical and biochemical barriers to drug absorption (11–13). Caco-2 cells have many properties similar to those of the enterocytes of the small intestine. They contain active transport and efflux proteins. According to the FDA, Caco-2 cell cultures can be used as an *in vitro* model in bioavailability/bioequivalence testing of highly soluble drugs that permeate cell layers well (12,14), together with *in vitro* dissolution tests. The capacity of a potential new drug to permeate absorptive cell layers can also be determined using Caco-2 cell cultures. One or several compounds with already known permeability properties need to be included. An *in vitro* absorption model of this kind has also

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ABBREVIATIONS: AP-BL, apical-to-basolateral; BL-AP, basolateral-to-apical; DMEM, Dulbeccós modified Eagle's medium; FBS, foetal bovine serum; HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MDR1, multidrug resistance gene; MES, 2-(*N*-morpholino)ethanesulfonic acid; MRP2, multidrug resistance associated protein; MTT, mitochondrial toxicity test; NEAA, non-essential amino acids; P_{app}, apparent permeability coefficient; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; TEER, transepithelial electrical resistance.

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been used to investigate whether the permeability of a drug is affected by other drugs administered at the same time (15–17).

The aim in the study reported was to determine whether extracts of food supplements (flax seed, purple loosestrife, Scots pine bark), berries (bilberries, cowberries, raspberries, all widely eaten in Finland) and some herbs (oregano, rosemary, sage), all of which could be used in preparing functional foods, affected the absorption of co-administered widely used highly permeable drugs verapamil, metoprolol, paracetamol, ketoprofen, and medium/low permeable furosemide. Because the extracts studied are complex mixtures, and drug concentrations at the site of absorption are high *in vivo*, 0.25 mM drug concentrations were chosen to demonstrate the possible interactions between extracts and drugs. Functional foods are foods in which one component or a combination of ingredients can improve the health and well being of a consumer beyond what is gained simply from its nutritional value (18). The effectiveness and safety of functional foods can only be established experimentally, *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Verapamil hydrochloride and ketoprofen were bought from ICN Biomedicals Inc. (Aurora, OH, USA) and metoprolol bitartrate from Sigma Chemical Co (St. Louis, MO, USA). Furosemide and paracetamol were donated by Orion Pharma (Espoo, Finland). D-[1-^{14}C]-mannitol (specific activity 59.0 mCi/mmol) was bought from Amersham Pharmacia Biotech UK Ltd. (Amersham, England), and rhodamine123 from Fluka Chemie GmbH (Buchs, Switzerland). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), heat-inactivated (+56°C for 30 min) fetal bovine serum (FBS), L-glutamine (200 mM), antibiotic mixture (10000 IU/ml penicillin G, 10000 μg/ml streptomycin), Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), and HEPES solution (10 mM) were bought from Gibco Invitrogen Corp. (Life Technologies Ltd. Paisley, Scotland). For the MTT assay, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was bought from Sigma Chemical Co (St. Louis, MO, USA), 1-sodium dodecyl sulfate (SDS) from Duchefa (Haarlem, The Netherlands), and isopropanol from Riedel-de Haën (Seelze, Germany). Dimethylsulphoxide (DMSO) was bought from ICN Biomedicals Inc. (Aurora, OH, USA), L-ascorbic acid from Sigma Chemical Co. (St. Louis, MO, USA), and methanol (technical grade) for use in extraction procedures from Algol (Espoo, Finland).

All of the organic solvents and other chemicals used in the extraction procedures and analyses were of analytical or chromatographic grade. Acetic acid was purchased from Riedel-de Haën (Seelze, Germany), acetonitrile and methanol from Rathburn (Walkerburn, Scotland), phosphoric acid from Merck (Darmstadt, Germany), and triethylamine (TEA) from Fluka Chemie AG (Buchs, Switzerland). Water was purified in an Alpha-Q water-purification system (Millipore, Molsheim, France). Ethanol (ETAX Aa 99.5%) was purchased from Primalco (Helsinki, Finland), and hydrochloric acid (HCl, 37%) from Riedel-de Haën (Seelze, Germany).

Preparation of Extracts

Flax seed and purple loosestrife were prepared according to (19). Extraction of 1 g of flax seed (*Linum usitatissimum* L., Linaceae) groats (Neomed Ltd., Somero, Finland) or 500 mg of dry powdered purple loosestrife (*Lythrum salicaria* L., Lythraceae) took place with 10 ml of 80% aqueous methanol. After stirring, the flax seed sample was sonicated for 10 min and the purple loosestrife sample for 5 min, and then centrifuged for 10 min (1500 × *g*). The supernatant was collected and the plant material re-extracted twice. The obtained extracts were concentrated by rotary evaporation (Rotavapor R110 Büchi Labortechnik, Staufen, Germany) with the water-bath temperature not exceeding 35°C, and freeze-dried. The extract residues were rinsed using 100% methanol (HPLC grade) into test tubes, which were then centrifuged. The supernatants were concentrated under nitrogen streams, freeze-dried and stored at +4°C until assay.

The Scots pine bark extract was prepared by freeze-drying 100 ml of a commercial Scots-pine-bark aqueous extract (*Pinus sylvestris* L., Pinaceae). For 1 g of extract, yielding 430 mg of dry extract, 0.3 g of bark and phloem are used, according to the manufacturer, Ravintorengas Oy, Siikainen, Finland, which kindly provided the samples.

Extracts of bilberries (*Vaccinum myrtillus* L., Ericaceae), cowberries (*Vaccinum vitis-idaea* L., Ericaceae), and raspberries (*Rubus idaeus* L., Rosaceae), of Finnish origins, purchased from a local supplier, were prepared by adding 30 ml of deionized water, 50 ml of ethanol, and 50 mg of L-ascorbic acid to 10 g of whole berries. The samples were homogenized using an Ultra Turrax mixer (T25B, Janke & Kunkel GmbH & Co. KG, IKA Labortechnik, Staufen, Germany).

Glycosidic fractions of the extracts were prepared by placing the samples for 2 h in a shaker. Each resulting homogenate was filtered into a volumetric flask and the volume was adjusted to 200 ml with an ethanol-water mixture (1:1). The obtained extracts were concentrated by rotary evaporation with the water-bath temperature not exceeding 35°C, and freeze-dried.

To obtain aglycone fractions, homogenates were hydrolyzed by boiling them with 20 ml of 6 M HCl under reflux for 2 h, after which they were allowed to cool to room temperature. The samples were filtered (Whatman No. 1, Whatman International Ltd., Maidstone, England) and the residues washed twice with 2 × 20 ml of an ethanol-water mixture (1:1). Each filtrate was placed in a volumetric flask and the volume adjusted to 200 ml with an ethanol-water mixture (1:1). The obtained extracts were concentrated by rotary evaporation with the water-bath temperature not exceeding 35°C, and freeze-dried.

The herb extracts were prepared according to (20). Briefly, 50 g of herb material (oregano (*Origanum vulgare* L., Lamiaceae), rosemary (*Rosmarinus officinalis* L., Lamiaceae) or sage (*Salvia officinalis* L., Lamiaceae), Paulig Group Ltd., Helsinki, Finland) was extracted with 500 ml of deionized water using a European Pharmacopoeia (Ph. Eur.) hydrodistillation apparatus for 2 h. The water was removed from the flask, 300 ml of fresh water were added and the mixture was left to boil for 1 h. The water-extraction fractions were combined, filtered through qualitative No. 4 Whatman filter paper (Whatman International Ltd., Maidstone, England), freeze-

dried (Heto LyoPro 3000, Allerød, Denmark) and stored at +4°C until use.

Determination of Total Phenolics

Quantities of total phenolics in the extracts were determined using the Folin-Ciocalteu procedure (21). The diluted samples (100 μl, three replicates) of the dry extracts were placed in 10 ml volumetric flasks, 0.5 ml of undiluted Folin-Ciocalteu's reagent was added and, after 1 min, 1.5 ml of sodium carbonate (20% w/v) was added and the volume made up to 10 ml with water. The contents of the flasks were mixed and allowed to stand for 1 h at 25°C. Absorbances were measured at 760 nm and compared to a previously obtained gallic-acid calibration curve. Total phenolic content was expressed as gallic-acid equivalents (GAE) in mg/g of dry material.

Preparation of Test Solutions

Verapamil, metoprolol, ketoprofen, paracetamol, furosemide, and rhodamine were dissolved in HBSS (pH 7.40) to concentrations of 0.5 mM. After dissolution, the pH levels of the final solutions were checked, and corrected, if necessary. All of the freeze-dried herb and food supplement extracts were dissolved in HBSS (pH 7.40) to final concentrations of 20.0, 2.0, 0.2, and 0.02 mg/ml. The glycosidic berry extracts were diluted with HBSS to concentrations of 2.0, 0.2, and 0.02 mg/ml. The aglycone fractions of the berry extracts were first dissolved in DMSO or ethanol and these solutions were then diluted with HBSS to final concentrations of 0.2, 0.02, and 0.002 mg/ml. The final DMSO or ethanol concentrations were 50 mg/ml. All of the berry extract solutions were kept at +4°C in darkness until use. At the start of each experiment, the extract solutions and drug solutions were combined (1:1), and the pH levels of the final test solutions were measured.

Cell Culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were seeded at 6.8×10^4 cells/cm^2 on to polycarbonate filter membranes with pore sizes of 0.4 μm and growth areas of 1.1 cm^2 in clusters of 12 wells (Corning Costar Corp., Cambridge, MA, USA). The cells were grown in a medium consisting of DMEM containing 4.5 g/l of 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 incubator (BB 16 gas incubator, Heraeus Instruments GmbH, Hanau, Germany) in an atmosphere of 5% CO_2 and 95% air, at 95% relative humidity. The growth medium was changed three times a week until time of use. 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.

MTT Toxicity Test

MTT is a tetrazolium salt that is cleaved by mitochondrial succinate dehydrogenases in living cells, yielding dark-blue formazan. Damaged or dead cells exhibit reduced or zero dehydrogenase activity. This colorimetric assay can be used to determine cell viability (mitochondrial activity) by measuring the extent of formazan formation after solubilization of the living material (22). Briefly, Caco-2 cells were seeded on to 96-well tissue culture plates (Costar Corp., Cambridge, MA, USA) 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) 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 in a multiwell scanning spectrophotometer Model 550 Microplate reader (Bio-Rad, Tokyo, Japan). Results ($n = 8$) were expressed as percentages of the control value (cells treated with HBSS only).

Permeability Experiments

All of the permeability experiments were performed under "sink conditions", meaning that amounts of compound transported to the acceptor chamber did not exceed 10% of amounts in the donor compartment. The permeabilities of verapamil, metoprolol, ketoprofen, paracetamol, mannitol, furosemide and rhodamine123 across Caco-2 cell monolayers were studied in an apical-to-basolateral (AP-BL) direction at an apical and basolateral pH of 7.40.

Before the permeability experiments, the cell monolayers had been washed twice with HBSS containing 10 mM HEPES, pH 7.40. After washing, the cells were allowed to come to equilibrium in the transport buffer for 30 min. Transepithelial electrical resistance (TEER) was measured using a Millicell ERS Voltohmmeter (Millipore Corp., Bedford, MA, USA). Cell monolayers with TEER values below 250 Ω were not used. The apical solution was changed to HBSS containing the drugs and/or the extracts. Samples were obtained after 15, 30, 45, 60, and 90 min by moving the cell monolayers to a new receiver well containing fresh HBSS. All of the transport experiments were conducted in triplicate. After each experiment, the cell cultures were washed once with HBSS, pH 7.4, and TEER values were measured. If values were below 220 Ω, the cell monolayers were further incubated with HBSS, and electrical resistance was measured again after 60 min. Samples were kept at –22°C until analysis.

Monolayer integrity was also determined using ^{14}[C]-mannitol (a paracellular marker molecule) at the same time as the permeability studies. Three cell monolayers were studied using only mannitol (controls). In some cases, ^{14}[C]-mannitol was included in the donor solutions with the drugs studied.

When the test solutions were prepared, the pH levels of solutions containing drug compounds and berry extracts, both glycosidic and aglycone fractions, were lower than 7.40. These reductions in pH levels of the solutions would probably have affected transport of the drugs across the Caco-2 cell monolayers. The effects of minor changes in pH level on the abilities of verapamil, metoprolol, paracetamol, ketoprofen and mannitol to permeate across Caco-2 cell monolayers were therefore determined. Briefly, the drugs were dissolved in HBSS at pH 6.50; 6.75; 6.90; 7.00; 7.15; 7.25; and 7.40 to concentrations of 0.25 mM. Once dissolution was complete, pH levels of the final test solutions were checked, and corrected, if necessary. Permeability experiments were conducted as described above.

After the berry extracts had been combined with the drugs, pH levels of the resultant solutions were measured. In a first set of experiments pH levels were not corrected. In a second set pH levels were adjusted to 7.40.

Determination of Transported Drugs

Drug concentrations in the receiver compartments were determined using HPLC (Waters Millennium, Milford, USA) and a Waters 486 Tunable Absorbance Detector, or Waters 470 Scanning Fluorescence Detector (furosemide detections), a Waters 717 plus Autosampler, and a Waters 510 pump. The mobile phase during determination of verapamil and metoprolol contained acetonitrile (33%), deionized water (45%), methanol (22%), TEA (0.03%) and 85% phosphoric acid (0.04%) (λ = 237 nm for verapamil, 200 nm for metoprolol). For determination of paracetamol, the mobile phase was composed of deionized water (65%), methanol (35%) and acetic acid (0.4%) (λ = 245 nm). For determination of ketoprofen the mobile phase consisted of acetonitrile (50%) and 0.03% phosphoric acid (50%) (λ = 254 nm), and for determination of furosemide of acetonitrile (40%) and 0.08 M phosphoric acid pH 1.8 (60%) (excitation-λ = 233 nm emission-λ = 389 nm). A μBondapak C_{18} reversed-phase column (300 × 3.9 mm; 10 μm, Waters, USA) with a C_{18} guard column (Waters, USA) was used, with a flow rate of 1 ml/min (paracetamol) or 1.5 ml/min (verapamil, metoprolol, ketoprofen, and furosemide).

For determination of ^{14}[C]-mannitol, 100 μl samples were removed from the receiver compartments, 4 ml of a scintillation cocktail (Optiphase HiSafe 2, Wallac Fisher Chemicals, Loughborough, England) were added, and ^{14}[C]-activities were measured by liquid scintillation counting, using a WinSpectral 1414 Liquid Scintillation Counter (Wallac, Turku, Finland). Rhodamine123 concentrations in samples were determined by Wallac Victor 1420 Multilabel Counter (PerkinElmer Inc. USA) with excitation-λ 435 nm and emission-λ 535 nm, and 1.0 s detection time.

Data Analysis

Cumulative amounts of drugs transported across Caco-2 cell monolayers were calculated from concentrations measured in the receiver (basolateral) compartments. Apparent permeability coefficients, P_{app} (cm/s), were calculated using the equation:

$$P_{app} = \Delta Q/(\Delta t \cdot A \cdot C_o),$$

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_o is the initial concentration of the compound in the donor (apical) compartment. Results reported are average P_{app} (cm/s) values ± SD (n = 3). Mass balance was determined from the sum of the cumulative amount transported and the amount remaining in the donor compartment in relation to the initial amount in the donor compartment. Typically, 75–105% of initial drug content was accounted for. It would therefore seem that the compounds studied were not retained in cellular structures or adsorbed on to the plastic equipment.

The P_{app} values relating to drugs with extracts (samples) 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.

The results were confirmed statistically using 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, USA). Significance level of 5% was used.

RESULTS AND DISCUSSION

Mitochondrial Enzyme Activity (MTT Test)

The effects of the drugs studied, of DMSO and of the extracts, on their own and in combination with solutions of the drugs, were tested on Caco-2 cell monolayers before the transport experiments.

DMSO, used to enhance the solubilities of the extracts, exhibited marked toxic effects at the higher concentrations (Table I). It increased mitochondrial enzyme activity shortly before the marked toxic effects appeared (viability clearly exceeded 100%). At concentrations of DMSO exceeding 50 mg/ml, slightly increased enzyme activity was measured. This was succeeded by toxic effects. The aglycone berry extracts were therefore dissolved in just 50 mg/ml DMSO.

No toxic effects in relation to mitochondrial enzyme activity were seen with any of the extracts at concentrations between 0.2 and 2.0 mg/ml, though slight toxic effects were seen with the sage and oregano extracts at 20 mg/ml (Table I). In the permeability experiments, the concentrations of the extracts of flax seeds, purple loosestrife, Scots pine, bilberries, cowberries, raspberries, oregano, rosemary and sage, were 0.01, 0.1, and 1.0 mg/ml. The aglycone fractions of extracts of bilberries, cowberries and raspberries exhibited no toxicity at the concentrations tested (0.002–0.2 mg/ml) (data not shown). In the subsequent experiments the concentrations of aglycone berry extracts used were 0.001, 0.01, and 0.1 mg/ml. Verapamil, metoprolol, paracetamol, ketoprofen, furosemide and rhodamine were found to be nontoxic at concentrations between 0.05 and 0.5 mM (data not shown).

Determination of Total Phenolics

Amounts of total phenolics in plant material varied widely, from 26 to 335 mg GAE/g of dry weight (dw) (Table II). Use of the Folin-Ciocalteu procedure indicated that the extracts contained anthocyanins, flavonols, hydroxybenzoic and hydroxycinnamic acid derivatives, and flavan-3-ols and some other phenolic compounds, such as tannins (23,24).

The used flax seed extract contained some phenolic compounds (26 mg GAE/g dw). Flax seeds mainly contain oils, proteins, carbohydrates and mucilage (Table II). The main phenolics in the purple loosestrife have been reported to be castalagin, valoneic acid, isoorientin, orientin, vescalagin and ellagic acid (19). The total phenolics in our extract was determined to 42 mg GAE/g dw. The Scots pine bark extract contained 112 mg GAE/g dw of phenolic compounds (proanthocyanidins, phenolic acids, catechin and kaempferol glycosides according to literature) (25).

The glycosidic fractions of the berry extracts contained about 10% of the phenolics present in the aglycone fractions. The ratio glycosidic/aglycone total phenolics in bilberry, cowberry, and raspberry extracts was 0.14, 0.08, and 0.13, respec-

Table I. The MTT Test: Caco-2 Cells (5.0×10^4 Cells/Well) Were Exposed to the Compounds Studied for 60 Min[a]

Compound	Concentration (mg/ml)	Enzyme activity (%)
DMSO	0.5	104 ± 5
	1.0	98 ± 4
	10	97 ± 7
	20	102 ± 3
	50	106 ± 2
	100	113 ± 10
	200	116 ± 10
	300	50 ± 7
	400	2.5 ± 1
Flax seed extract	0.02	115 ± 26
	0.2	89 ± 9
	2	99 ± 11
	20	106 ± 7
Purple loosestrife extract	0.02	110 ± 16
	0.2	110 ± 3
	2	129 ± 33
	20	106 ± 17
Scots pine bark extract	0.02	129 ± 15
	0.2	93 ± 6
	2	109 ± 3
	20	120 ± 5
Bilberry extract	0.02	87 ± 10
	0.2	91 ± 24
	2	88 ± 10
	20	103 ± 8
Cowberry extract	0.02	124 ± 14
	0.2	132 ± 23
	2	131 ± 14
	20	126 ± 7
Raspberry extract	0.02	120 ± 4
	0.2	124 ± 12
	2	121 ± 16
	20	112 ± 16
Sage extract	0.02	101 ± 31
	0.2	107 ± 25
	2	105 ± 13
	20	79 ± 7
Oregano extract	0.02	88 ± 28
	0.2	97 ± 21
	2	97 ± 22
	20	77 ± 16
Rosemary extract	0.02	105 ± 19
	0.2	107 ± 20
	2	111 ± 22
	20	94 ± 15

[a] Results are mean percentages ± SD (n = 8) of control value (100%) (no exposure).

tively (Table II). The main phenolics reported to be present in Finnish bilberries and cowberries are hydroxycinnamic acids (63%) and flavonols (70%), respectively (26,23). Amounts of hydroxybenzoic acids in these berries are relatively low. Ellagic acid and ellagic tannins are the main phenolic compounds reported in raspberries (85–88%).

Levels of phenolics found in the herb extracts studied ranged from 149 to 185 mg GAE/g dw (Table II). Rosmarinic acid was confirmed as the main phenolic constituent in such extracts (20).

Permeability Experiments

Transport of Drug Compounds

P_{app} values following apical loading with 0.25 mM solutions of verapamil, metoprolol, paracetamol, ketoprofen, furosemide, 0.025 mM rhodamine123, and mannitol (controls) were 28.1 ± 2.4 (n = 36); 28.8 ± 2.5 (n = 36); 29.8 ± 2.3 (n = 30); 22.7 ± 0.9 (n = 33), 0.23 ± 0.02 (n = 9), 1.21 ± 0.11 (n = 9), and 0.22 ± 0.03 (n = 18) (10^{-6} cm/s), respectively.

The permeability of metoprolol and paracetamol (passive transcellular diffusion) was high, and mannitol (passive paracellular diffusion) was low. Monocarboxylic acid transporter is assumed to enhance ketoprofen absorption in the human intestine and Caco-2 cells under acidic conditions in the apical compartment (27). Verapamil, a substrate for the P-glycoprotein (P-gp) efflux system (28,29) has a high absorptive (AP-BL) permeability at concentrations between 0.1 and 0.5 mM, most probably because active efflux is saturated. According to Doppenschmitt *et al.* (30), the absorption-reducing effect of P-gp is most probably concealed by the high passive permeability of verapamil, and its high affinity for P-gp at concentrations (e.g., 0.25 mM) that are relatively high but still normally used. Rhodamine123 is considered as a substrate for P-gp efflux system (31), and its absorptive permeability is medium low. For the low permeability drug furosemide, paracellular route might play an important role in absorption, as well as P-gp or MRP2 efflux pumps or OAT transporters (32).

Effect of Extracts on the Permeability of Co-administered Drugs

Food Supplements. The permeabilities of all of the compounds studied, except verapamil and rhodamine123, were decreased when flax seed extract was also present (Fig. 1, furosemide and rhodamine data not shown). Ketoprofen and metoprolol transport was most affected. The minor changes in mannitol diffusion and high TEER values after the experiments indicate that paracellular spaces were tightly closed. Flax seeds contain health-promoting factors, such as phytoestrogen and lignan precursors, flax seed oil, proteins, mucilage and phenolic acids (Table II) (33). Flax seed oil contains α-linolenic acid, which is believed to have cancer-preventive properties (34). The decreases in the abilities of the drugs to permeate across Caco-2 cell monolayers may be caused by the mucilage, soluble fibers and lignans present in the extract. Decreases could be significant if any of the drugs studied were ingested at the same time as large quantities of flax seeds. Flax seeds are used as laxatives and the laxative effect depends on the fiber content. Fluids are adsorbed on to the fibers and the intestinal contents swell. This could result in adsorption of other dissolved compounds, for example, drugs, on to flax seed fibers, and decreased absorption.

Purple loosestrife extract, which contains several flavonoids, anthocyanins, phenolic acids, tannins and phthalates (Table II) (19) decreased the permeability of the basic drugs verapamil and metoprolol, and increased the abilities of the acidic compounds to permeate across Caco-2 cell monolayers (Fig. 1). This could be a result of the presence of tannins or phenolic acids (gallic acid and methyl gallate) in the extract, which could increase acidity at sites of absorption. The permeability of furosemide or rhodamine123 was not signifi-

Table II. Phenolic Compounds Reported for the Plant Species Studied and the Total Phenolics Determined for the Plant Extracts Used in This Study

Species	Chemical components	References	Total phenolics (mg of GAE/g dw)[a]
Flax, seed *Linum usitatissimum* L.	Oil (35–45%), proteins (20–25%), mucilage (6–10%), polysaccharides: D-galacturonic acid, D-galactose, L-rhamnose, D-xylose, minor monosaccharides)	(56) (57) (58)	26.2
	Phenolic acids: caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid, 4-hydroxybenzoic acid, sinapic acid, syringic acid, vanillic acid	(59) (60)	
	Cyanogenic glycosides: linamarin, linustatin, neolinustatin		
	Lignans: secoisolariciresinol diglucoside		
Purple loosestrife *Lythrum salicaria* L.	Flavonoids: isoorientin, orientin, vitexin, isovitexin	(61)	42.1 (25)
	Anthocyanins: cyanidin-3-galactosidase, malvidin-3,5-diglucoside	(62)	
	Phenolics: chlorogenic acid, *p*-coumaric acid, dilactoneisochloro genic acid, ellagic acid, gallic acid, methyl gallate, valoneic acid	(19) (63)	
	Tannins: castalagin, pedunculagin, lythrines, vescalagin, etc.	(64)	
	Pthalates, sterols, terpenes		
Scots pine, bark *Pinus sylvestris* L.	Flavonoids: catechin 3-β-glucopyranoside, dihydroconiferin, kaempferol 3-β-rhamnopyranoside, taxifolin-3-*O*-β-glucopyranoside	(65) (37)	111.9
	Phenolic acids: *p*-coumaric acid β-glucopyranoside, ferulic acid β-glucopyranoside, *p*-hydroxybenzoic acid β-glucopyranoside, protocatechuic acid, vanillic acid β-glucopyranoside		
	Tannins: proanthocyanidins B1, B2, B3; lignans		
Bilberry *Vaccinum myrtillus* L.	Flavonoids: catechin, epicatechin, kaempferol, myricetin, quercetin, quercetin-*O*-glycoside	(66) (67)	G 37.8[b] A 279.0[c]
	Phenolic acids: caffeic acid, *p*-coumaric acid, ellagic acid, ferulic acid, *p*-hydroxybenzoic acid	(26) (68)	
	Anthocyanins: cyanidin-3-glycosides, delphinidin-3-glycosides, peonidin-3-glycosides, petunidin-3-glycosides, malvidin-3-glycosides	(23)	
	Tannins: ellagitannins, procyanidin B2		
Cowberry *Vaccinum vitis-idaea* L.	Flavonoids: Kaempferol, myricetin, quercetin, quercetin-*O*-glycosides	(69)	G 25.7[b]
	Phenolic acids: caffeic acid, *p*-coumaric acid, ellagic acid, ferulic acid, *p*-hydroxybenzoic acid	(67) (26)	A 317.5[c]
	Anthocyanins: cyanidin-3-glycosides, delphinidin-3-glycoside		
	Tannins: ellagitannins		
Raspberry *Rubus idaeus* L.	Flavonoids: catechin, epicatechin, kaempferol, kaempferol-3-glycosides myricetin, quercetin, quercetin-3-glycosides	(70) (26)	G 44.7[b] A 335.0[c]
	Phenolic acids: caffeic acid, *p*-coumaric acid, ellagic acid, ferulic acid, *p*-hydroxybenzoic acid	(71) (23)	
	Anthocyanins		
	Tannins: ellagitannins		
Oregano *Origanum vulgares* L.	Flavonoids: apigenin, luteolin-7-*O*-glucoside	(72)	149.0 (20)
	Phenolic acids: caffeic acid, *o*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, rosmarinic acid, vanillic acid	(73)	
	Carvacrol (phenol)		
	Benzoates and hydroxycinnamates		
Rosemary *Rosmarinus officinalis* L.	Flavonoids: apigenin, cirsimaritin, hispidulin, naringin	(74)	185.0 (20)
	Phenolic acids: caffeic acid, carnosic acid, *p*-coumaric acid, ferulic acid, gentisic acid, *p*-hydoxybenzoic acid, protocatechuic acid, rosmarinic acid, syringic acid, vanillic acid	(75)	
Sage *Salvia officinalis* L.	Flavonoids: apigenin, cirsimaritin, hispidulin, luteolin, luteolin-7-*O*-glucoside, scutellarein	(76) (74)	166.0 (20)
	Phenolic acids: caffeic acid, carnosic acid, *p*-coumaric acid, ferulic acid, gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, rosmarinic acid, sinapic acid, syringic acid, vanillic acid	(75) (77) (42)	
	Carnosol (diterpene), methyl carnostate		

[a] Determined as gallic acid equivalents (GAE) according to the Folin-Ciocalteu procedure. Values are means of three analyses.
[b] Glycosidic fraction (G).
[c] Aglycone fraction (A).

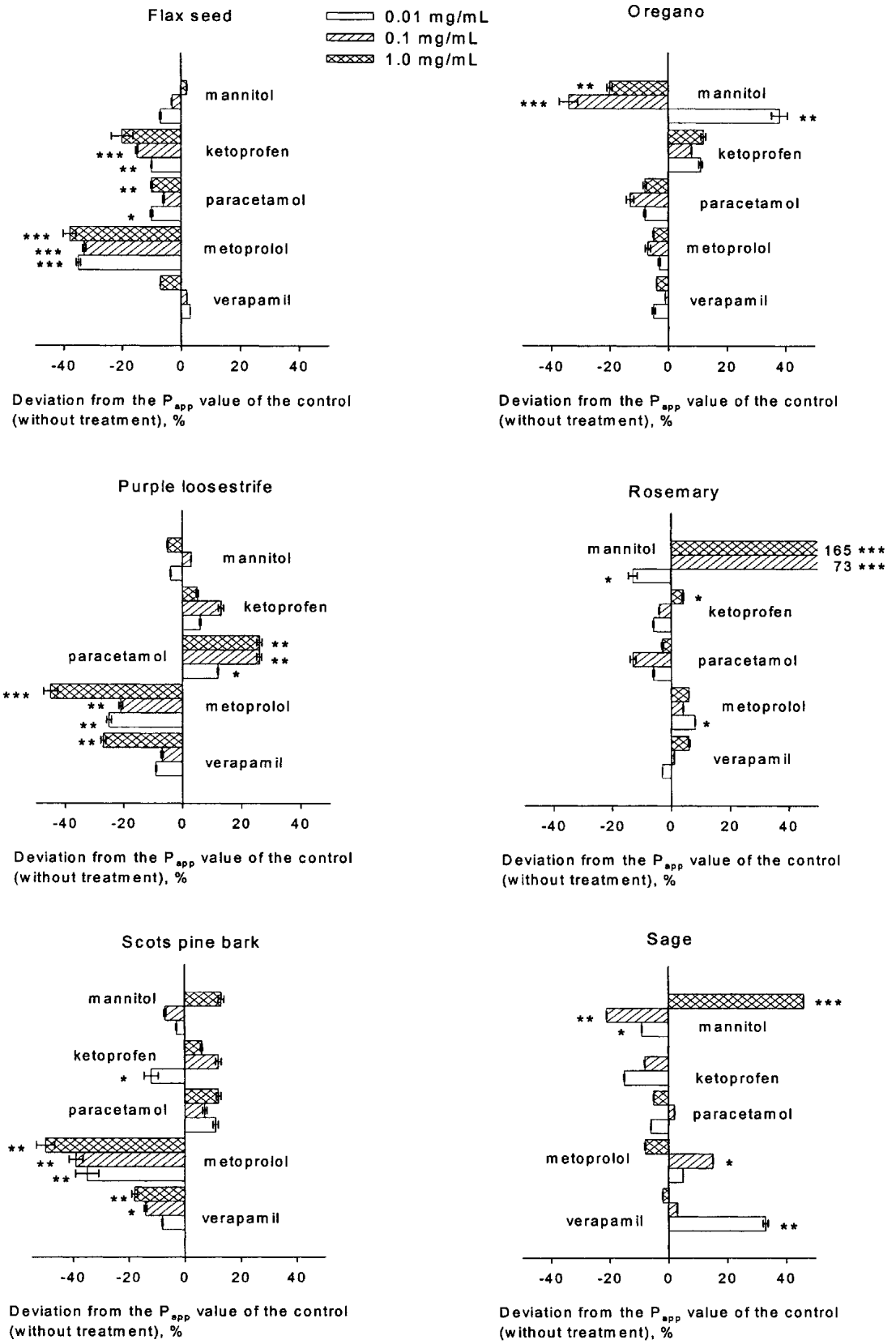

Fig. 1. Changes in permeabilities of verapamil, metoprolol, paracetamol, ketoprofen, and mannitol in the presence of various concentrations of extracts of food supplements and herbs across Caco-2 cell monolayers. Values are mean percentage differences (n = 3) ± SDs from the permeabilities of the compounds across Caco-2 cell monolayers in the absence of the extracts (control values) ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cantly affected (data not shown). Purple loosestrife, in the form of a decoction or liquid extract, is traditionally used to treat diarrhea. A registered extract product of *Lythrum salicaria* is on the market for this purpose in Europe. The flowers can be used to abate the symptoms of hemorrhoids (35). They have been shown to have antifungal activity and some antibacterial activity, *in vitro* (36).

Scots pine bark extract had only minor effects on permeability of the acidic compounds studied but more pronounced effects on the transport of verapamil and metoprolol (Fig. 1). The permeability of furosemide was slightly enhanced (11%), and rhodamine diffusion decreased (25%) (data not shown). Scots pine bark has been reported to contain a mixture of flavonoids and many phenolic acids and tannins, mainly proanthocyanidins (Table II). Taxifolin-3-*O*-β-glucopyranoside and procatechuic acid have been found to have antioxidant properties (37). They have been reported to influence various physiologic events, such as inflammation, platelet aggregation, immune responses, vasorelaxation and capillary permeability (38–39). The decreases in the abilities of metoprolol and verapamil to permeate across Caco-2 cell monolayers at high extract concentrations could again have been caused by the effects of the tannins or phenolic acids present in the extract, which could increase acidity at sites of absorption. Absorption of these basic drugs could be affected *in vivo* if high doses of a pine bark preparation were ingested.

Berries When pH levels of solutions of berry extracts and drugs were measured before the permeability experiments, the buffering capacity of HEPES in HBSS was insufficient to keep the pH of the sample solutions to 7.40. Low pH levels were observed in all cases. The glycosidic fraction of raspberry extract (1.0 mg/ml) had the greatest effect in lowering pH (to 7.00).

If large quantities of acidic food or acidic food supplement extracts were ingested over a brief period, pH levels at sites of absorption could be reduced, affecting the transport of contemporaneously ingested drugs, depending on the pKa value of the drug. According to the pH partition theory, acidic compounds are transported more effectively in acidic environments. Basic compounds behave in the opposite way. The effects depend on the pK_a of a compound and pH at the absorption site. When the pH of the apical transport medium was reduced from 7.40 to 7.00 and that of the basolateral medium was kept at 7.40, the permeabilities of the drugs across Caco-2 cell monolayers in the absence of any berry extract changed to some extents (Fig. 2), but mannitol diffusion was not affected (data not shown). According to the pH partition theory, the relative contributions of the unionized form of bases with a pK_a of 8.7 (verapamil) and 9.7 (metoprolol) are about 5% and 0.5% at pH 7.40, respectively. The corresponding values at pH 7.00 are 2% and 0.2%. The permeabilities of verapamil and metoprolol at an apical pH of

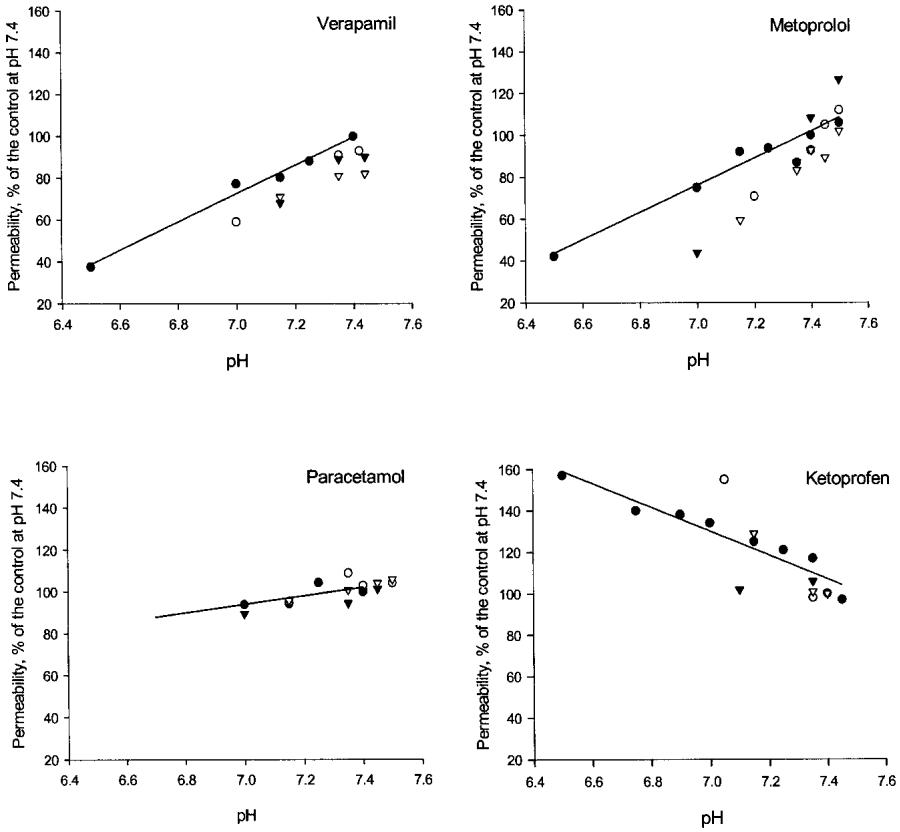

Fig. 2. Relative permeabilities (in %) of verapamil, metoprolol, paracetamol, and ketoprofen as a function of apical pH in the absence of any extract (controls, ● + regression line) and in the presence of various concentrations of extracts of bilberries (○), cowberries (▽), and raspberries (▼). The lowest pH values always correspond to the highest extract concentrations. All values are the averages of values from three experiments and are expressed as percentage permeabilities across Caco-2 cell monolayers, where 100% permeability is the permeability at pH 7.40.

7.00 were reduced to about 80% of the control value at pH 7.40. This finding is consistent with findings in experiments by Neuhoff *et al.* (40). The relative contribution of the unionized form of ketoprofen (pK_a 5.9) is about 3.1% at an apical pH of 7.40 and about 7.4% at an apical pH of 7.00**.** The permeability of ketoprofen was 1.35 times greater at pH 7.00 than at pH 7.40. The relative contributions of the unionized form of the very weak acid paracetamol (pK_a 9.5) are 99.2% and 99.7% at pH 7.4 and 7.0, respectively. This should not lead to any significant differences in permeabilities at the studied pH levels. No significant differences were in fact observed (94% of control value at pH 7.40).

Relative P_{app} values for the drugs studied in the presence and absence of berry extracts at identical pH levels were the same, except in the case of metoprolol, where there was a slight difference (Fig. 2). After the effects of pH on the transport of the drugs studied were recognized, the experiments with berry extracts were repeated; correcting the pH after the drug and berry extract solutions had been combined (Fig. 3). At pH 7.4, the presence of glycosidic and aglycone fractions of the berry extracts affected the permeabilities of the studied drugs only slightly overall. The presence of cowberry extract resulted in slight increases in the abilities of verapamil and metoprolol to permeate across Caco-2 cell monolayers (Fig. 3), although it had reduced these permeabilities at lower pH levels (Fig. 2). Only cowberry showed some, but not dose-dependent, effects on transport of ketoprofen.

Herbs. Neither oregano nor rosemary extracts hardly affected the permeabilities of the highly permeable drugs studied across Caco-2 cell monolayers (Fig. 1). With sage extracts at a low concentration (0.01 mg/ml) the ability of verapamil to permeate Caco-2 cell monolayers was enhanced (by 33%). Higher concentrations (0.1 and 1.0 mg/ml) had scarcely any effects on permeability of the highly permeable drugs studied. The permeability of furosemide was enhanced by oregano, rosemary, and sage extracts (1 mg/ml) 35%, 365% and 13%, respectively (Table III). Rhodamine permeability was slightly decreased by rosemary (21%). Mannitol diffusion was affected by all of the herb extracts (Fig. 1). The relative P_{app} value (percentage enhancement or inhibition in relation to control P_{app} value) was increased by 38% by the presence of oregano extract at 0.01 mg/ml but decreased by 34% and 20% in the presence of oregano extracts at concentrations of 0.1 and 1.0 mg/ml, respectively. Various compounds present in oregano extract (Table II) might affect the TEER of Caco-2 monolayers, and thus the paracellular diffusion of mannitol. Also co-administration of other drugs might affect the tightness of the monolayers. A dose-dependent increment of TEER values indicating a tight closure of paracellular spaces at low verapamil concentrations (0.1–0.3 mM) has been reported by Sakai *et al.* (41), but higher verapamil concentrations (0.7–1.0 mM) caused dose-dependent drop of TEER and increment in paracellular diffusion. The presence of rosemary and sage extracts markedly enhanced mannitol diffusion at high extract concentrations. The enhancement was associated with a reversible lowering of TEER values (195 ± 25 Ωcm^2 10 min after the experiment, and 275 ± 15 Ωcm^2 60 min after the experiment). The findings strongly suggest that the herb extracts caused partial opening of paracellular spaces between Caco-2 cells. Small changes in tightness of paracellular spaces can significantly affect the permeability of hydrophilic paracellularly permeated small mol-

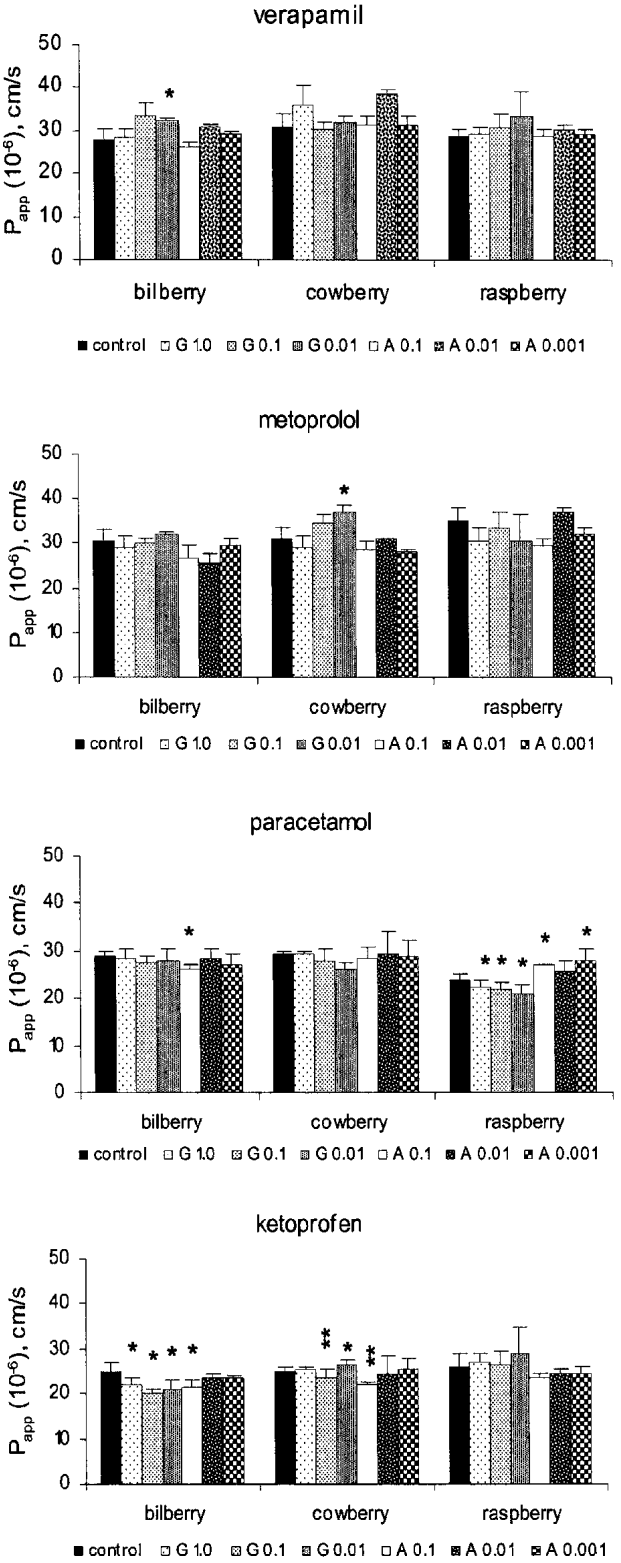

Fig. 3. Apparent permeability (P_{app}) of verapamil, metoprolol, paracetamol, and ketoprofen in the absence (control) or presence of extracts of bilberries, cowberries, and raspberries after correction of pH to 7.40. All the values are averages (n = 3) ± SDs. G = glycosidic fraction; A = aglycone fraction. Concentration is in mg/ml. *$p < 0.05$, **$p < 0.01$.

Table III. Effects of the Herbs Oregano, Rosemary, and Sage on the Permeability of Furosemide and Rhodamine123 Across Caco-2 Cell Monolayers

	P_{app} (10^{-6}) cm/s					
Drug	Control n = 9	Oregano n = 3 1 mg/ml	Rosemary n = 6 1 mg/ml	n = 3 0.1 mg/ml	n = 3 0.01 mg/ml	Sage n = 3 1 mg/ml
Furosemide	0.23 ± 0.02	0.31 ± 0.02[b]	0.84 ± 0.01[c]	0.24 ± 0.01	0.23 ± 0.02	0.26 ± 0.02
Rhodamine123	1.21 ± 0.11	1.33 ± 0.09	0.95 ± 0.09[a]	0.98 ± 0.09[a]	1.06 ± 0.10	1.29 ± 0.09

Results are mean values ± SD.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

ecules. However, because the absorptive area in the paracellular space is relatively small, substantial opening of the paracellular space would seem not to influence the overall permeabilities of the highly permeable compounds studied across Caco-2 cell monolayers.

Both oregano and rosemary extracts caused increment in furosemide permeability (Table III). Rosemary extract (1 mg/ml) enhanced apparent permeability of furosemide almost 4-fold. As considerable part of absorbed furosemide is diffused paracellularly (32), the strong enhancement in its permeability can partly be explained by opening of tight junctions between Caco-2 cells, because mannitol permeability was also enhanced (165-fold, Fig. 1). The effects of these extracts on rhodamine permeability were detectable, but not as significant.

Rosmarinic acid, the main phenolic component of all of the herb extracts, has antioxidative properties (42). Other phenolic compounds present in the herbs studied are flavonoids (apigenin, naringin and luteolin), and phenolic acids (caffeic, ferulic and vanillic acids) (Table II).

The various phenolic compounds in plant extracts can affect the permeabilities of drugs across Caco-2 cell monolayers. Phenolic compounds are normally absorbed to greater or lesser extents, depending on their structures. Apigenin, which is present in all of the herbs (Table II), is a flavonoid analog of genistein. The bioavailabilities of genistein and its glucoside analog genistin in human beings have been reported to be low, because of extensive first-pass metabolism or bacterial degradation at sites of absorption (43,44). The permeabilities of genistein and apigenin aglycones across Caco-2 cell monolayers have been reported to be high in both the AP-BL and BL-AP directions. However, the aglycones may undergo extensive phase-II metabolism in enterocytes. Walle *et al.* (45) have suggested that the poor bioavailability of genistin may result from the presence of multidrug-resistance-associated protein-2 (MRP2) on the apical surfaces of Caco-2 cells. According to Castro and Altenberg (46), genistin absorption may be reduced by the presence of P-glycoprotein (P-gp, MDR1). If apigenin were a substrate of P-gp, it could affect the permeability of other P-gp substrates, such as verapamil or rhodamine, across Caco-2 cell monolayers, if the binding site at P-gp was same for both substrates (47).

Naringin, one of the major flavonoids in grapefruit juice and rosemary extracts (Table II), has been reported to enhance the oral and systemic availabilities of a variety of drugs (48). Effects on systemic availability were explained by acute inhibition of the MDR1 efflux pump system that exists in various parts of the small intestine (46,49).

Methanolic extracts of rosemary have been reported to inhibit P-gp efflux activity in breast-cancer cell cultures that over-expressed P-gp (50). Competitive binding to P-gp site(s) *in vitro* was determined after 1 h to 4 h of exposure to the extract. It was demonstrated by intracellular accumulation of the commonly used chemotherapeutic agents doxorubicin and vinblastine. The mechanism underlying the phenomenon is believed to be the ability of the rosemary extract to alter the activity of P-gp but not its expression. The aqueous rosemary extract used in our experiments did not enhance the ability of the P-gp substrates verapamil or rhodamine to permeate Caco-2 cell monolayers. The differences in findings may be explained by differences in composition of the rosemary extracts, reflecting different growing conditions of the plants, differences in extraction procedures and the relatively brief time of exposure to the rosemary extract in our experiments. Plouzek's group used a cell culture that over-expressed P-gp. Our Caco-2 cell culture does not over-express P-gp.

All of the berries studied contain quercetin and its glycosides, kaempferol and myricetin (Table II) (26). Bilberries and raspberries also contain catechin and epicatechin. Quercetin and its glycosides are present to major extents in vegetables and fruits. A diet rich in the flavonoid quercetin has been reported to confer protection against coronary disease and stroke (51). Results of animal studies and *in vitro* findings suggest that such flavonoids also protect against cancer (e.g., Ref. 52). The per oral absorption of quercetin glucosides is low and variable (0–24%) in human beings (53), depending on the sugar concerned (54). Transport of quercetin and its glycosides across Caco-2 cell monolayers has been studied by Walgren *et al.* (8). They found that the AP-BL permeability of quercetin across Caco-2 cell monolayers was more than 10 times greater than the permeability of mannitol (a low permeability marker molecule) and five times lower than the permeability of propranolol (a high permeability marker molecule). The permeabilities of quercetin 4-glucoside and quercetin 3,4-diglucoside were fairly detectable. The BL-AP permeabilities of quercetin, quercetin 4-glucoside and quercetin 3,4-diglucoside were respectively twice, 80 times and four times the AP-BL permeability. In further experiments with verapamil as a P-gp inhibitor and MK-571 as a MRP2 inhibitor was observed that quercetin glycosides were actively taken up by the sodium-dependent glucose transporter SGLT1 in Caco-2 cells but transported back to the donor

compartment by the MRP2 efflux pump present on the apical surfaces of Caco-2 cells (9,10). These results indicate that the permeability of verapamil should not be affected by foods containing quercetin, because verapamil has not been found to be a substrate of MRP2.

Catechin and epicatechin are present, with other flavonoids, in substantial amounts in green tea, red wine, chocolate, many fruits and some berries (including bilberries and raspberries) (55). Epicatechin has no AP-BL permeability across Caco-2 cell monolayers. Its BL-AP efflux has been found to be slightly higher than that of mannitol. In the presence of MK-571, efflux fell by half. Absorption was detectable but slightly less than that for mannitol, indicating that outflow of epicatechin could be effected by MRP2. Consequently, its presence should not affect the permeability of verapamil as our experiments also showed.

CONCLUSIONS

The extracts of food supplements, berries and herbs studied were not toxic in relation to Caco-2 cell monolayers. The presence of extracts of flax seeds decreased the permeabilities of all of the drugs studied except verapamil and rhodamine123, possibly because of the mucilage, soluble fibers and lignan in the extracts. The presences of extracts of purple loosestrife and pine bark reduced the permeabilities across Caco-2 cell monolayers of basic drugs, possibly as a result of interactions with tannins or phenolic acids in the extracts. The glycosidic and aglycone fractions of extracts of berries were associated mainly with pH-related changes in the permeabilities of the drugs studied. The permeabilities of metoprolol, ketoprofen and paracetamol across Caco-2 cell monolayers were mostly unaffected by the presence of the herb extracts studied. The permeability of verapamil, but not rhodamine was increased by 33% in the presence of sage extract (0.01 mg/ml). Furosemide permeability was strongly increased by the presence of 1 mg/ml rosemary extract. Increases in the permeabilities of mannitol were always associated with low TEER values, but the effect was reversible. Our results indicate in general that all of the extracts of berries and herbs studied may be ingested contemporaneously with highly permeable drugs verapamil, metoprolol, ketoprofen and paracetamol. However, if high doses of extracts were administered with low permeable drugs, effects on drug permeabilities could not be excluded.

The effects/interactions between food supplements and drugs are more complicated *in vivo* than they are *in vitro*. At the site of absorption *in vivo*, many digestive enzymes and variable pH conditions might degrade food supplements and/or drugs. Metabolism at the site of absorption might affect the bioavailability of them. However, *in vitro* absorption experiments are important to gain valuable first-hand information about the interactions between health-promoting products and common drugs.

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