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Polyphenols from Evening Primrose (*Oenothera paradoxa*) Defatted Seeds Induce Apoptosis in Human Colon Cancer Caco-2 Cells

Sylwia Gorlach,[*,†,∥] Waldemar Wagner,[§,∥] Anna Podsędek,[†] Dorota Sosnowska,[†] Jarosław Dastych,[§] and Maria Koziołkiewicz[†]

[†]Institute of Technical Biochemistry, Technical University of Lodz, Stefanowskiego 4/10, 90-924 Lodz, Poland

[§]Laboratory of Cellular Immunology, Institute of Medical Biology, Polish Academy of Sciences, Lodowa 106, 93-232 Lodz, Poland

Supporting Information

ABSTRACT: Polyphenols extracted from evening primrose seeds (industrial waste product) were studied as apoptosis inducers in human colorectal adenocarcinoma Caco-2 and HT-29 cell lines and in rat normal intestinal IEC-6 cells. The extract dose-dependently inhibited the growth of Caco-2, HT-29, and IEC-6 cells. However, nuclear DNA fragmentation characteristic of apoptosis was observed only in Caco-2. After 72 h of incubation with the extract at 150 μM gallic acid equivalents (44.1 μg extract/mL), Caco-2 cell numbers decreased to 19% of control and 48.8% of the cells were identified by flow cytometry as apoptotic. Under the same conditions only 8% of HT-29 cells and 12.6% of IEC-6 cells exhibited hypodiploid DNA content. The effects of the extract and its fractions on phosphatidylserine exposure and cell membrane integrity were assessed by high content screening image cytometry. The fractions strongly and dose-dependently reduced Caco-2 cell numbers, whereas HT-29 and IEC-6 cells were affected to lesser extents.

KEYWORDS: polyphenols, evening primrose, *Oenothera paradoxa*, apoptosis, colon cancer, Caco-2, cell cycle

INTRODUCTION

Evening primrose (*Oenothera* sp.) waste defatted seeds are generated in large quantities in the process of oil pressing. They are a rich source of phenolics, which are present or are planned to be introduced on the market as a diet supplement in some countries. The *Oenothera paradoxa* Hudziok variety is used as a source of oilseeds in Poland, and polyphenols investigated in our study were obtained from seeds of this variety. The polyphenolic profile of the *O. paradoxa* seeds has recently been investigated, although it is still incomplete. Three phenolic acids (gallic, ellagic, and caffeic) together with several flavonoids have been identified in the seeds so far.[1] The isolated flavonoids belong to flavanols ((+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, procyanidin dimer B3), hydrolyzable tannins (pentagalloylglucose (PGG), oenothein B), and flavonols (quercetin and its glucuronide).

In contrast to a large number of papers on the bioactivities of individual polyphenols, studies with polyphenol-rich extracts are still relatively scarce, except for grape, apple, and cocoa extracts, which have been widely investigated in the past decade. Investigations on evening primrose polyphenol extracts and their biological activities have been limited to a few in vitro studies[1−4] and one in vivo study, which demonstrated a strong hypocholesterolemic effect of dietary *O. paradoxa* cake extract in rats.[5] A polyphenol extract from *Oenothera biennis* defatted seeds was shown to trigger apoptosis in Ehrlich ascites tumor cells,[2] and in another study the selective proapoptotic activity of the extract toward human and murine bone marrow-derived tumor cells was attributed to a gallic acid-containing phenolic fraction.[3] Polyphenol extracts obtained from the defatted seeds of *O. paradoxa* Hudziok variety were shown to significantly and dose-dependently inhibit metallopeptidase activity, and the inhibitory activity was largely attributed to the dominating compounds in the extracts, namely, PGG and procyanidins with different degrees of polymerization.[1] Later, the extracts were also demonstrated to exert proapoptotic activity toward human skin melanoma HTB-140 cells.[4]

Systemic bioavailability of high molecular weight (MW) polyphenols, such as condensed and hydrolyzable tannins, is generally very low.[6] However, their concentrations in the lumen of the gastrointestinal tract are likely to reach the lower millimolar range, as estimated in ref 7. On the other hand, ellagic acid (a low MW product that was generated by colonic microflora from punicalagin, a hydrolyzable tannin) triggered apoptosis in human colon cancer Caco-2 cells.[8] Importantly, even high doses of punicalagin were reported as nontoxic to rats after repeated oral administration for 37 days.[9] Furthermore, ellagic acid metabolites (urolithins) were shown to arrest Caco-2 cell growth at S and G2/M cell cycle phases.[10] The gastrointestinal tract is the most accessible for ingested polyphenols, with low MW compounds being metabolized and absorbed in the stomach and small intestine, whereas high MW ones were reported to reach the colon or its nonphysiological counterpart, an ileostomy bag.[6] It is worth highlighting that low MW microbial degradation products of high MW polyphenols can be absorbed into the bloodstream and exert their activities at the systemic level in addition to acting locally in the colon.[11,12]

Nevertheless, the gastrointestinal tract remains the first site of action for all ingested polyphenols. We therefore decided to

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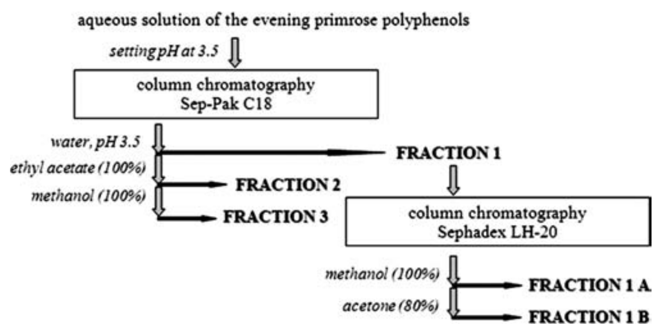

Figure 1. Scheme of evening primrose (*Oenothera paradoxa*) seed polyphenol extract fractionation.

extract polyphenols from the evening primrose waste defatted seeds and assess their apoptosis-inducing activity in two human colorectal adenocarcinoma cell lines, Caco-2 and HT-29. In parallel, we conducted experiments with rat normal intestinal IEC-6 cells.

MATERIALS AND METHODS

Materials. Evening primrose (*O. paradoxa* Hudziok) waste defatted seeds were obtained from Agropharm S.A. (Tuszyn, Poland). All reagents used in our study were special grade commercial products purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. All solvents were of analytical or HPLC grade. Sep-Pak C18 chromatographic columns were obtained from Waters Corp. (Milford, MA). Cell culture media and supplements were obtained from Sigma-Aldrich, except for penicillin and streptomycin (Polfa Tarchomin, Tarchomin, Poland) and amphotericin B (Biochrom AG, Berlin, Germany). Annexin V−Alexa Fluor 647 conjugate, YO-PRO-1, and Hoechst-33342 were purchased from Invitrogen Corp., Molecular Probes, Inc. (Eugene, OR).

Polyphenol Extraction from Evening Primrose Defatted Seeds. Evening primrose seeds obtained in the process of oil pressing were milled and additionally defatted with hexane. Polyphenols were extracted from the milled and defatted seeds with the use of a 70% aqueous solution of ethanol at room temperature. The first two extractions lasted 30 min each and were followed by one 15 min extraction. The ratio of plant material to ethanol solution was 1:10 (w/v) in the first two extractions and 1:5 (w/v) in the third one. The resulting extracts were centrifuged (15 min, 4000 rpm), and then the pooled extracts were concentrated under vacuum at <40 °C. The obtained aqueous solution of the evening primrose polyphenols was lyophilized and stored at −20 °C prior to further analyses. The resulting preparation is referred to as the polyphenol extract (polyphenol preparation) henceforth in this work.

Fractionation of the Evening Primrose Polyphenol Extract. The polyphenol extract was fractionated by solid phase extraction with the use of Sep-Pak C18 followed by column chromatography on a Sephadex LH-20 bed,[13] as presented in Figure 1. The starting material was the aqueous solution of the evening primrose polyphenols obtained according to the procedure described above. Prior to the fractionation, the pH of the solution was set to 3.5. The Sep-Pak C18 chromatographic columns (10 g) were consecutively washed with methanol and water (pH 3.5), and the above-mentioned aqueous polyphenol extract was applied on the columns. The first fraction was obtained by washing the Sep-Pak with water (pH 3.5). The absorbed polyphenols were subsequently washed with ethyl acetate (fraction 2) and methanol (fraction 3). The corresponding fractions from seven fractionations were pooled. Fractions 2 and 3 were concentrated until dryness at <30 °C and dissolved in 96% aqueous solution of ethanol. The polyphenols of the aqueous fraction 1 were separated by column chromatography on Sephadex LH-20 bed. The bed was initially suspended in a 25% aqueous solution of methanol. After 24 h, the column (2.0 × 32 cm) was filled with the suspension and washed with water at a rate of 2 mL/min with the use of a peristaltic pump. Fraction 1 was loaded on the column, and the absorbed polyphenols were eluted first with methanol (thus obtaining fraction 1A) and then with 80% aqueous solution of acetone, which resulted in fraction 1B. The pooled methanol fractions (1A) and the pooled acetone fractions (1B) obtained from seven fractionations on Sephadex LH-20 were concentrated under vacuum until dryness and subsequently dissolved in a 96% aqueous solution of ethanol.

Flavanol Isolation from Evening Primrose Defatted Seeds. Evening primrose flavanol preparation was obtained by extraction combined with precipitation.[14] The milled and additionally hexane-defatted seeds were treated with 90% aqueous solution of acetone in a ratio of 1:2.5 (w/v). Then the pooled extract was treated twice with trichloromethane (in the ratio of 1:1, v/v). The organic phase was removed. The aqueous phase was saturated with sodium chloride, and flavanols were extracted with ethyl acetate. Water was removed by freezing, and flavanols were precipitated from ethyl acetate with the use of chloroform. The resulting preparation is subsequently referred to as the flavanol preparation in this work.

Characterization of Polyphenol and Flavanol Preparations. The polyphenol and flavanol preparations were characterized in terms of total polyphenol content expressed as (+)-catechin, gallic acid, or ellagic acid;[15] total flavanol content expressed as (+)-catechin;[16] total proanthocyanidin content expressed as cyanidin;[17] total hydrolyzable tannin content expressed as methyl gallate;[18] mean degree of proanthocyanidin polymerization (DP);[19] and in vitro antioxidant activity.[20−22] The preparations were also analyzed using high-performance liquid chromatography (HPLC)[23] and matrix-assisted laser desorption−ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Antioxidant Activity Measurement. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{•+}$) scavenging activity was determined following the procedure described by Re et al.[20] Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a synthetic analogue of α-tocopherol) was used as a standard, and the capacity of free radical scavenging was expressed as Trolox equivalent antioxidant capacity (TEAC), that is, in micromoles of Trolox per milligram of the polyphenol or flavanol preparations or per milligram of total polyphenols. Ferric reducing antioxidant power (FRAP) was assessed according to the method of Benzie and Strain,[21] and the antioxidant activity was expressed as TEAC. Determination of polyphenol efficiency in inhibition of linoleic acid oxidation in an oil/water emulsion was carried out according to the procedure described in ref 22, and the efficiency was expressed as the inhibition time of carbonyl compound generation.

HPLC Analysis. Phenolics were analyzed by reversed-phase (RP)-HPLC with the use of a Eurospher-100 C18 column (5 μm, 250 × 4.6 mm) (Knauer, Berlin, Germany). Binary mobile phase was prepared according to the method of Dyrby et al.[23] Solvent A was water and formic acid in a ratio of 90:10 (v/v), respectively. Solvent B was water, acetonitrile, and formic acid in a ratio of 40:50:10 (v/v/v), respectively. The separation of phenolics was performed using the following gradient program with a flow rate of 1 mL/min: 0 min, 88% A + 12% B; 26 min, 70% A + 30% B; 40−43 min, 0% A + 100% B; 48−50 min, 88% A + 12% B. The phenolics were divided into three groups on the basis of the wavelength at which the maximum of UV−vis absorption was observed. Flavan-3-ols and hydroxybenzoic acid derivatives were quantified at 280 nm and expressed as (+)-catechin equivalents, hydroxycinnamic acid derivatives at 320 nm as chlorogenic acid equivalents, and flavonols at 360 nm as rutin equivalents.

MALDI-TOF MS. Mass analyses were performed using a Voyager-Elite MALDI-TOF mass spectrometer (Per-Septive Biosystems Inc., Framingham, MA) equipped with delayed extraction. Typical conditions

included 20 kV acceleration voltage and a nitrogen laser pulse (wavelength 337 nm). High-resolution positive- and negative-ion mode spectra were recorded in reflection mode. 2,5-Dihydroxybenzoic acid was used as a matrix.

Cell Culture and Treatment with Evening Primrose Polyphenols. Human colorectal adenocarcinoma Caco-2 and HT-29 cell lines were obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wroclaw, Poland). Rat normal small intestine epithelial IEC-6 cells were purchased from the Health Protection Agency (London, U.K.). Caco-2 and HT-29 cells were cultured in MegaCell MEM supplemented with 3% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1.25 μg/mL amphotericin B. IEC-6 cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum, 0.1 IU/mL insulin, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1.25 μg/mL amphotericin B. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. For the polyphenol treatment, harvested cells were suspended in media of the composition given above (except for amphotericin B, which was excluded) and seeded on 96-well plates (for image cytometry) or 6-well plates (for flow cytometry and DAPI staining). Cells were seeded at densities that ensured approximately 80% confluency in controls at the end of a 5 day experiment. After 48 h, culture medium was replaced by fresh medium and cells were incubated with selected concentrations of the evening primrose preparations for indicated time. The polyphenol preparation (polyphenol extract) and flavanol preparation were dissolved in 70% ethanol solution in deionized water; the polyphenol extract fractions were dissolved in 96% aqueous solution of ethanol. All of the solutions were further diluted with the culture medium, so that the final concentrations of the organic solvents were <0.01% (v/v). The polyphenol extract was tested at total polyphenol concentrations ranging from 10 to 150 μM gallic acid equivalents (GAE), which corresponds to 1.7−25.5 μg GAE/mL or 2.9−44.1 μg extract/mL. The polyphenol extract fractions and the flavanol preparation were tested at 50, 75, and 100 μM GAE. Controls for the polyphenol treatment were incubated with ethanol.

High Content Screening (HCS). The effects of evening primrose polyphenol preparations on selected cellular functions were tested by means of HCS image cytometry, where three parameters of cell viability, phoshpatidylserine exposure on the outer surface of plasma membrane, cell membrane integrity, and cell nuclei morphology, were analyzed simultaneously at single-cell resolution. Cells of three selected cell types were incubated without or with increasing concentrations of polyphenols on a 96-well microplate. Half an hour before the end of 48 or 72 h incubations Hoechst-33342 solution in PBS was added to the culture medium at the final concentration of 1 μg/mL. Then, cells were washed with cold PBS followed by a wash with a buffer enabling annexin V binding to phosphatidylserine (10 mM HEPES, 140 mM NaCl, 2,5 mM $CaCl_2$, pH 7.4). The solution of annexin V−Alexa Fluor 647 conjugate and YO-PRO-1 was prepared in the above-mentioned buffer and used for staining of cells for 15 min at room temperature. After the incubation, cells were washed with the same buffer and the 96-well microplate was immediately analyzed in an ArrayScan V^{TI} HCS Reader (Thermo Fisher Scientific, Inc.). Acquired photographs were analyzed with Multiparameter Cytotoxicity Bioapplication V3 software (Cellomics BioApplications, Cellomics, Inc.) to quantify the loss of cell membrane asymmetry detected as an increase in phosphatidylserine available for annexin V binding, changes in cell membrane permeability, and changes in cell nuclear morphology.

Cell Cycle Analysis and Apoptosis Detection by Flow Cytometry. Cell cycle parameters and the percentage of cells with hypodiploid DNA content (apoptotic cells) were determined by means of flow cytometry on the basis of propidium iodide (PI) staining. Following 48 or 72 h of incubation with or without polyphenols cells were washed twice with PBS, harvested by trypsinization, combined with the cells pelleted from the culture medium, and further washed with ice-cold PBS. Cell fixation was carried out with the use of 70% ice-cold ethanol aqueous solution for 1 h on ice and fixed cells were stored at −20 °C until the analysis. Before analysis cells were washed twice with PBS and incubated in a phosphate−citrate buffer (0.192 M Na_2HPO_4, 4 mM citric acid, pH 7.8) containing 0.25 mg/mL heat-inactivated ribonuclease A and 33 μg/mL propidium iodide for 1 h at room temperature in the dark. After the incubation, cell DNA content was determined on the basis of fluorescence intensity observed in FL2 with the use of a flow cytometer (Cytomics FC 500 MPL, Beckman Coulter, Inc.), and the numbers of cells in each part of the histogram representing <2*N*, 2*N*, 2*N*−4*N*, and 4*N* DNA content were determined using CXP software (Beckman Coulter, Inc.).

Cell Cycle Analysis by Image Cytometry. Cell cycle was analyzed on the basis of DNA content determined by Hoechst-33342 fluorescence intensity. After 72 h of incubation with or without polyphenols, cells were washed with PBS and fixed in situ (i.e., on 96-well plates) with 4% formaldehyde solution in PBS for 30 min at room temperature. The solution was prepared from paraformaldehyde directly before usage, and its pH was set to 7.4. After fixation, cells were washed with PBS and stained with Hoechst-33342 (1 μg/mL) for 30 min at room temperature in the dark. After additional washing with PBS, cells were analyzed in an ArrayScan V^{TI} HCS Reader. Photographs of 16 microscopic fields were acquired per each well, and the images of Hoechst-33342-stained cells were analyzed using the Cell Cycle Bioapplication V3 program to determine the number of cells in each phase of cell cycle.

Detection of Apoptosis by 4′,6-Diamidino-2-phenylindole (DAPI) Staining. Apoptotic cells were identified on the basis of nuclear morphology changes visualized by DAPI staining. After 72 h of incubation with or without polyphenols, cells were harvested, washed with PBS, fixed with 1% formaldehyde solution in PBS (pH 7.4) for 30 min at room temperature followed by ice-cold 70% ethanol fixative for 1 h on ice, and stained with 1 μg/mL DAPI. Stained cells were observed under a fluorescence microscope at 400× magnification.

Statistical Evaluation of Data. Data are presented as the mean ± SEM or SD, as indicated in the figure captions. The number of independent experiments or the number of analyzed cells per well (*n*) is also given in the figure captions. For the results of HCS the statistical significance of differences between means was determined by one-way ANOVA followed by Holm Sidak test using SigmaStat 3.5 software (SPSS, Inc., Chicago, IL). For the remaining results nonparametric analysis (Kruskal−Wallis one-way ANOVA followed by Bonferroni test) was carried out with the use of Analyze-it software, v. 2.21 (Analyze-it Software, Ltd., Leeds, U.K.). p values of <0.05 were considered to be statistically significant.

RESULTS

Preparation, Fractionation, and Characterization of Evening Primrose Seed Polyphenols. Polyphenols and one of their subgroups (flavanols) were isolated from evening primrose defatted seeds, and the preparations were characterized in terms of composition (Table 1) and antioxidant activity (Table 1S in the Supporting Information). Total polyphenols were extracted with 70% aqueous solution of ethanol, and flavanols were isolated by means of a method combining extraction with precipitation. Due to the fact that the total polyphenol content expressed as GAE was between the content expressed as (+)-catechin equivalents and the content expressed as ellagic acid equivalents (the highest and the lowest, respectively) (Table 1), we decided to choose GAE for data presentation in the present work. The

Table 1. Characteristics of Evening Primrose Seed Polyphenol and Flavanol Preparations (n = 4 Measurements)

preparation	mg/g of preparation				mg/g of preparation			
	total polyphenols[a]	total flavanols[b]	proanthocyanidins[c]	DP[d]	hydroxybenzoic acids and flavanols[e]	hydroxycinnamic acids[f]	flavonols[g]	hydrolyzable tannins[h]
polyphenol	668.71 ± 15.76 578.15 ± 13.63 416.14 ± 9.80	190.02 ± 5.32	142.86 ± 4.05	2.85	645.42 ± 20.18	0	0	89.08 ± 8.13
flavanol	922.89 ± 31.42 830.09 ± 28.26 647.85 ± 22.05	422.46 ± 9.11	201.60 ± 7.82	2.45	851.46 ± 30.26	0	0	196.76 ± 13.31

[a] Total polyphenols determined according to ref 15 and expressed as (+)-catechin (first row), gallic acid (second row), and ellagic acid (third row). [b] Total flavanols determined according to ref 16 and expressed as (+)-catechin. [c] Proanthocyanidins determined according to ref 17 and expressed as cyanidin. [d] Mean degree of proanthocyanidin polymerization determined according to ref 19. [e] Hydroxybenzoic acids and flavanols determined by HPLC at 280 nm according to ref 23 and expressed as (+)-catechin. [f] Hydroxycinnamic acids determined by HPLC at 320 nm according to ref 23 and expressed as chlorogenic acid. [g] Flavonols determined by HPLC at 360 nm according to ref 23 and expressed as rutin. [h] Hydrolyzable tannins determined by HPLC according to ref 18 and expressed as methyl gallate formed from the tannins during acid hydrolysis in methanol environment.

Table 2. Characteristics of the Evening Primrose Seed Polyphenol Extract Fractions (n = 4 Measurements)[a]

fraction	mg/fraction				DP
	total polyphenols	polyphenols (HPLC; 280 nm)	total flavanols	proanthocyanidins	
1A	127.07 ± 9.66	120.95 ± 8.81	25.81 ± 0.32	18.39 ± 0.15	3.08
1B	81.81 ± 4.74	72.40 ± 4.23	16.65 ± 1.16	17.37 ± 0.33	3.59
2	659.75 ± 26.61	627.60 ± 23.21	209.38 ± 9.08	76.18 ± 1.81	1.71
3	1284.48 ± 57.35	1248.30 ± 42.36	283.67 ± 6.71	249.69 ± 12.68	3.23
Σ 1−3	2153.11	2069.25	535.51	361.63	

[a] The flavanol and proanthocyanidin concentrations were determined with the same methods and expressed in the same units as in Table 1, whereas the total polyphenols and polyphenols determined by HPLC at 280 nm[23] are expressed as (+)-catechin equivalents.

waste seeds proved to be a good source of both polyphenols and flavanols: 42% of total flavanols and 20% of proanthocyanidins (oligomeric and polymeric flavanols) were detected in the flavanol preparation (Table 1). Both isolates contained hydrolyzable tannins determined as methyl gallates formed during acid hydrolysis of tannins in methanol. The hydrolyzable tannins constituted 9% of the polyphenol extract and 20% of the flavanol preparation. According to HPLC analysis, the group of compounds showing maximum absorbance at 280 nm (flavanols and/or hydroxybenzoic acids) predominated in both preparations. Neither hydroxycinnamic acids nor flavonols were detected in the isolates by HPLC analysis.

The evening primrose polyphenol extract was fractionated by solid phase extraction with the use of Sep-Pak C18 columns followed by column chromatography on a Sephadex LH-20 bed (Figure 1). Four fractions were obtained and characterized in terms of composition (Table 2) and antioxidant activity (Table 2S in the Supporting Information). Ninety percent of total polyphenols and approximately 87% of total flavanols (including proanthocyanidins) were recovered in the fractionation process. According to the authors of the fractionation method,[13] fractions 1A, 1B, and 3 should contain tannins, whereas low MW polyphenolic compounds (such as free phenolic acids, their derivatives, and flavonoids) should be present in fraction 2. Mean DP values obtained in the present work (Table 2) indicate that the evening primrose polyphenols were separated as stated above. Low MW compounds with mean DP = 1.71 (fraction 2) constituted 31% of the total polyphenol content of the recovered material (ΣF 1−3). The fraction contained 39% of total flavanols and 21% of total proanthocyanidins of ΣF 1−3. The highest polyphenol content was detected in fraction 3 (60% of total polyphenols, 53% of total flavanols, and 69% of proanthocyanidins of ΣF 1−3). Furthermore, the amounts of total flavanols and proanthocyanidins in fraction 1B imply that it did not contain flavanol monomers.

The antioxidant activity of the evening primrose preparations was studied with three methods ($ABTS^{\bullet+}$ scavenging activity, FRAP, and linoleic acid oxidation assay) (Table 2S in the Supporting Information). On the basis of the results the highest efficiency was attributed to fraction 2, which contained mainly low MW compounds (mean DP = 1.71). Although the remaining fractions had similar mean DP values (3.08, 3.59, and 3.23), they differed in TEAC values and in the extent of inhibition of linoleic acid oxidation. The antioxidative activities of all the fractions expressed in μmol of Trolox/fraction correlated with their total polyphenol content as well as with their total flavanol content. In the case of the results expressed in μmol of Trolox/mg of polyphenols, one can conclude that under the experimental conditions the compounds present in the flavanol isolate were more efficient antioxidants than those constituting the polyphenol extract (Table 1S in the Supporting Information). As far as the third method of antioxidant activity measurement is

Table 3. Tentatively Identified Peaks (TIPs) in the Positive- and Negative-Ion Mode MALDI-TOF Mass Spectra of the Evening Primrose Seed Polyphenol Extract and Fraction 2 (Low MW Fraction) (Analysis Carried Out for *m*/*z* Ratio up to 1000)

			extract		fraction 2	
no.[a]	compounds to which TIPs are ascribed[b]	MW[c]	*m*/*z*	TIPs	*m*/*z*	TIPs
1	quinic acid	192			215.0	$[M + Na]^+$
2	resveratrol	228	267.1	$[M + K]^+$		
3	(epi)afzelechin	274			297.2	$[M + Na]^+$
4	(+)-**catechin** and/ or (−)-**epicatechin**	290	291.0	$[M + H]^+$	291.1	$[M + H]^+$
5	caffeoylmalic acid	296			296.2 297.2	$[M - e]^+$ $[M + H]^+$
6	**ellagic acid** and/ or **quercetin**	302	340.9	$[M + K]^+$	303.1 301.1	$[M + H]^+$ $[M - H]^-$
7	monogalloylglucose	332	331.1	$[M - H]^-$		
8	resveratrol glucoside	390	413.3 429.2	$[M + Na]^+$ $[M + K]^+$	429.4	$[M + K]^+$
9	epiafzelechin gallate	426	465.2	$[M + K]^+$		
10	ellagic acid xyloside	434			457.4	$[M + Na]^+$
11	(−)-**epicatechin gallate** and/ or catechin gallate	442	465.2	$[M + Na]^+$	441.2	$[M - H]^-$
12	(epi)gallocatechin gallate	458	497.2	$[M + K]^+$		
13	A-type procyanidin dimer	576	576.9	$[M + H]^+$	599.5 577.2	$[M + Na]^+$ $[M + H]^+$
	procyanidin B3 (B-type procyanidin dimer)[d]	578				
14	B-type (epi)gallocatechin dimer	610	610.6	$[M - e]^+$		
15	trigalloylglucose	636	636.6	$[M - e]^+$		
16	1,3,5-tricaffeoylquinic acid and/ or 3,4,5-tricaffeoylquinic acid	679	678.6	$[M - e]^+$		
17	hexahydroxydiphenyl (HHDP)-digalloylglucose	786			786.7 787.7	$[M - e]^+$ $[M + H]^+$
18	tetragalloylglucose	788			787.4	$[M - H]^-$
19	procyanidin trimer (2A)	862			861.9	$[M - H]^-$
20	procyanidin trimer (2B)	866	866.7	$[M - e]^+$	905.3[f]	$[M + K]^+$
21	B-type dimeric procyanidin digallate and/ or B-type dimer of (epi)catechin gallate (*/**) and/ or trimer composed of two (epi)catechins and one (epi)gallocatechin (2B) (*/***)	882	881.1	$[M - H]^-$	905.3[e]	$[M + Na]^+$
22	trimer composed of two (epi)gallocatechins and one (epi)catechin (1A, 1B)	896	896.7	$[M - e]^+$ or $[M + H]^+$		

Table 3. Continued

no.[a]	compounds to which TIPs are ascribed[b]	MW[c]	extract		fraction 2	
			m/z	TIPs	m/z	TIPs
23	**pentagalloylglucose**	940	939.4	$[M - H]^-$	939.5	$[M - H]^-$
24	procyanidin tetramer (2A, 1B)	1150	574.2	$[M - 2H]^{-2}/2$		

[a] The numbers are assigned to MALDI-TOF MS peaks in Figure 1S of the Supporting Information. [b] The names of compounds that have so far been reported as constituents of evening primrose (*O. paradoxa*) seeds [1] are in bold. [c] Approximate MW (the values are rounded to the nearest integer). The assignements of m/z values to ions of phenolic compounds are approximate due to the natural occurrence of carbon-13 (^{13}C) and to possible slight shifts of peak positions along the x-axis. [d] B(A) = interflavanoid bond of B(A)-type; xA, yB = mixed-type procyanidin molecule with xA- and yB-type interflavanoid bonds. [e] The m/z ratio of 905.3 may correspond to at least four kinds of ions: $[M + K]^+$ (where M corresponds to MW of a B-type procyanidin trimer); and/or $[M + Na]^+$ (where M corresponds to MW of a B-type dimeric procyanidin digallate); and/or $[M + Na]^+$ (where M corresponds to MW of a B-type dimer of (epi)catechin gallate); and/or $[M + Na]^+$ (where M corresponds to MW of a B-type trimer composed of two (epi)catechins and one (epi)gallocatechin).

concerned, both preparations inhibited the oxidation of the linoleic acid emulsion.

MALDI-TOF MS. The evening primrose extract and its fractions were qualitatively analyzed by MALDI-TOF MS (Table 3; selected mass spectra in the Supporting Information). In general, the MALDI-TOF mass spectra confirmed the presence of some compounds identified in previous studies[1] and revealed the presence of others, among them (epi)(gallo)catechin gallates, A-type procyanidin dimer, A- and B-type procyanidin trimers, procyanidin tetramer with 2A and 1B interflavanoid bonds, B-type dimeric procyanidin digallate and/or B-type dimer of (epi)-catechin gallate, and several phenolic acids as well as hydrolyzable tannins such as mono-, tri-, and tetragalloylglucose and hexahydroxydiphenyl(HHDP)-digalloylglucose. Ellagic acid xyloside and resveratrol together with its glucoside were also tentatively identified, among other phenolics. Interestingly, the results of this preliminary analysis suggest that, besides procyanidins, the extract may contain prodelphinidins and mixed proanthocyanidins, where procyanidin monomers are linked to prodelphinidin monomers. For instance, TIP 14 in Table 3 might correspond to a B-type (epi)gallocatechin (prodelphinidin) dimer. Similarly, TIP 21 might originate from a B-type trimer composed of two (epi)catechins and one (epi)gallocatechin, whereas a trimer composed of two (epi)gallocatechins and one (epi)catechin with B-type + A-type interflavanoid bonds might be represented by TIP 22. Because of the high biological activities exerted by fraction 2 (see below), identification of its components was important: the MS analysis tentatively confirmed the presence of low MW phenolics (including (epi)catechin, (epi)catechin gallate, ellagic acid, and/or quercetin) in this fraction.

Influence of Evening Primrose Polyphenols on Caco-2, HT-29, and IEC-6 Cell Growth and Nuclear Morphology. The number of cells attached to the microplate well bottom surface was assessed on the basis of Hoechst-33342 staining of cell nuclei after 48 and 72 h of incubation with evening primrose preparations. The polyphenol extract dose-dependently inhibited the growth of all cell types used in our study, with Caco-2 being the most sensitive to the treatment and IEC-6 slightly more resistant than HT-29. The decrease in cell growth was statistically significant for all cell types ($p < 0.001$). For Caco-2 the IC_{50} value was 75−100 μM GAE; that for HT-29, 125−150 μM GAE; and for IEC-6 the IC_{50} value was not reached in our study, with the cell number of 55% versus control at the highest concentration tested (Figure 2A). All of the fractions and the flavanol preparation dose-dependently inhibited Caco-2 and HT-29 cell growth, and Caco-2 cells were definitely more sensitive to the treatment (Figure 4A). The strongest growth-inhibitory effect toward Caco-2 was exerted by fraction 2 and the weakest by fraction 1A. Nuclear morphology changes characteristic of apoptosis (chromatin condensation and fragmentation) were observed only in the case of Caco-2 (data not shown).

Influence of Evening Primrose Polyphenols on Cell Cycle. Cell cycle phase distribution and percentage of apoptotic cells with hypodiploid DNA content were determined after 48 and 72 h of incubation with the evening primrose polyphenol extract by flow cytometry of PI-stained cells (Figure 3 and Figure 2S in the Supporting Information). The influence of the polyphenol extract fractions and the flavanol preparation on cell DNA content presented as the $2N/4N$ index was assessed by image cytometry after Hoechst-33342 staining of cell nuclei (Figure 5). The index represents the ratio of the number of cells in G0/G1 phase (DNA content = $2N$, where N corresponds to haploid DNA content) to the number of cells in G2/M phase (DNA content = $4N$). Figure 3B shows that the increase in the amount of apoptotic cells was evident only in the case of Caco-2. Forty-eight hours of incubation of the cells with the extract resulted in a statistically significant ($p < 0.001$ at 150 μM GAE), dose-dependent increase in the amount of apoptotic cells (from a mean value of 2.4% in control to 41.1% at the highest concentration tested) (Figure 3B). The increase in sub-G1 peak was accompanied by a decrease in G0/G1 and, as a consequence, a statistically significant decrease in $2N/4N$ index (Figure 3A). At lower concentrations of the extract extensive fluctuations of the index were noticeable. After additional 24 h, the amount of apoptotic cells increased further to a mean value of 48.8% versus 5.4% in control (Figure 3B). The flavanol preparation induced marked, dose-dependent changes in Caco-2 DNA content, whereas among the extract fractions the one denoted 2 evoked the strongest effect (Figure 5).

The extract did not cause a considerable increase in hypodiploid peak in HT-29 cells (Figure 3B). However, after 48 h of incubation there was a slight, dose-dependent increase in the G2/M peak accompanied by a statistically significant decrease in the S peak, which could result from a G2/M cell cycle arrest (Figure 3B). After an additional 24 h, the amount of apoptotic cells increased to a certain extent, which might be a consequence of the putative G2/M block. At higher concentrations of the extract there was a decrease in G0/G1 cell cycle phase, probably

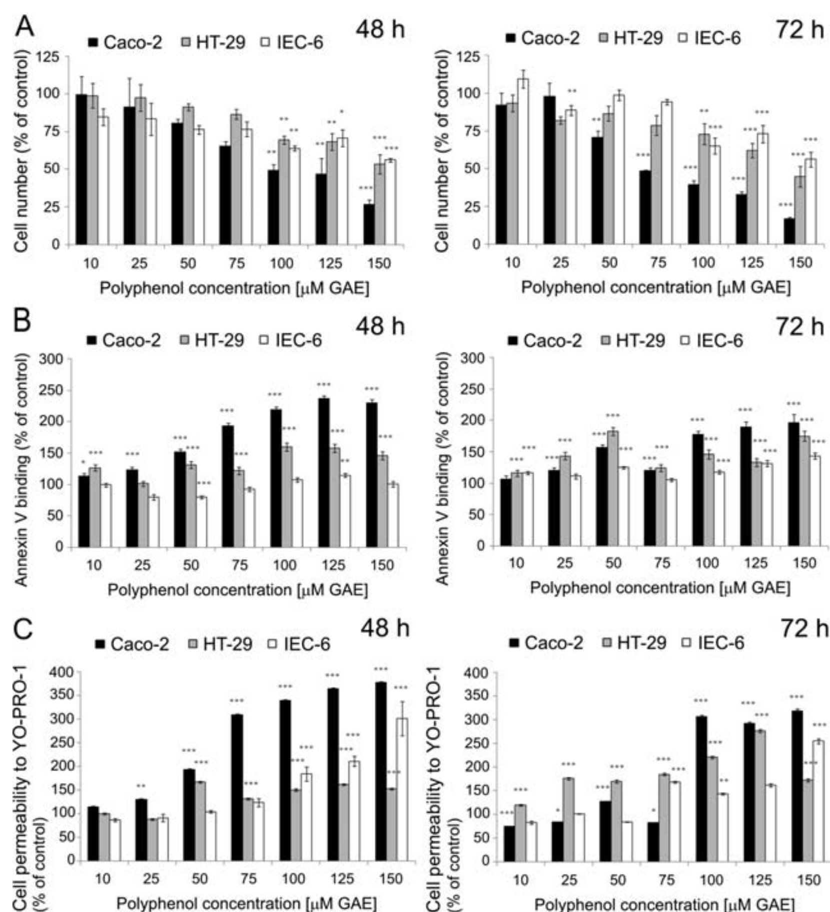

Figure 2. Influence of evening primrose seed polyphenol extract on Caco-2, HT-29, and IEC-6 cell number (A), annexin V binding (B), and cell membrane permeability to YO-PRO-1 (C) after 48 and 72 h of incubation. Cell number was determined by image cytometry, calculated as the sum of Hoechst-33342-stained cell nuclei counted in 16 microscopic fields per well. Annexin V−Alexa Fluor 647 conjugate and YO-PRO-1 fluorescence was measured in the HCS experiment. Data are expressed as the percentage of control. Each value represents the mean value ± SEM, $n = 3$ independent experiments (A) or $n = 3000-5400$ Caco-2 cells, $n \geq 5000$ HT-29 cells, $n = 500-1500$ IEC-6 cells (B and C). Significance of differences between means: ∗, $p < 0.05$, ∗∗, $p < 0.01$, ∗∗∗, $p < 0.001$ versus control.

originating from the above-mentioned arrest. As a result, one could observe a statistically significant, dose-dependent decrease in the $2N/4N$ index at higher concentrations tested (Figure 3A). All of the remaining preparations induced a dose-dependent decrease in the $2N/4N$ index albeit to a lesser extent than for Caco-2 (Figure 5). Fraction 2 was the most potent in this respect, as in the case of Caco-2.

After 48 h of incubation, the evening primrose polyphenol extract had no statistically significant influence on IEC-6 cell cycle, except for a very slight decrease in the G2/M peak at 75 and 150 μM. A slight, dose-dependent increase in the number of apoptotic cells could be observed, with a mean value of 8.4% at the highest concentration tested versus 3.6% in control (Figure 3B). There was also an increase in the G0/G1 peak accompanied by the above-mentioned slight decrease in G2/M population and, as a consequence, the value of the $2N/4N$ index augmented to a certain extent ($p < 0.05$) (Figure 3A). After an additional 24 h of incubation, there was some decrease in the G0/G1 peak accompanied by a very slight increase in both S and hypodiploid peaks (Figure 3B). The number of apoptotic cells reached 12.6% at the highest concentration tested versus 11.8% in control. The $2N/4N$ index value remained on a roughly constant level throughout the concentration range (Figure 3A). None of the remaining polyphenol preparations substantially influenced the index except for fraction 2, which caused a slight increase in the index value (Figure 5).

Influence of Evening Primrose Polyphenols on Cell Annexin V Binding. Alterations of the intrinsic cell membrane asymmetry characteristic of apoptosis were detected by HCS image cytometry after 48 and 72 h of incubation with the polyphenol extract on the basis of annexin V binding to phosphatidylserine exposed in the outer leaflet of cell membrane. In the case of Caco-2 there was a statistically significant ($p < 0.001$), dose-dependent increase in annexin V−Alexa Fluor 647 conjugate fluorescence intensity (up to 2.4-fold vs control), particularly for the shorter incubation time (Figure 4B). Among the remaining preparations the strongest effect was observed for fraction 2 and for the highest concentration of fraction 3 (Figure 4B). Annexin V binding by HT-29 cells was generally less pronounced than that by Caco-2 (Figure 4B). As in the case of Caco-2, fraction 2 exerted the strongest effect (Figure 4B). For IEC-6 cells there was a slight increase in fluorescence intensity at higher concentrations of the extract (Figure 2B). A moderate increase in annexin V binding was detected for 75 μM GAE flavanol preparation, as well as for fractions 1A, 1B, and 2 (at 50 and 75 μM GAE) (Figure 4B). At the highest concentration

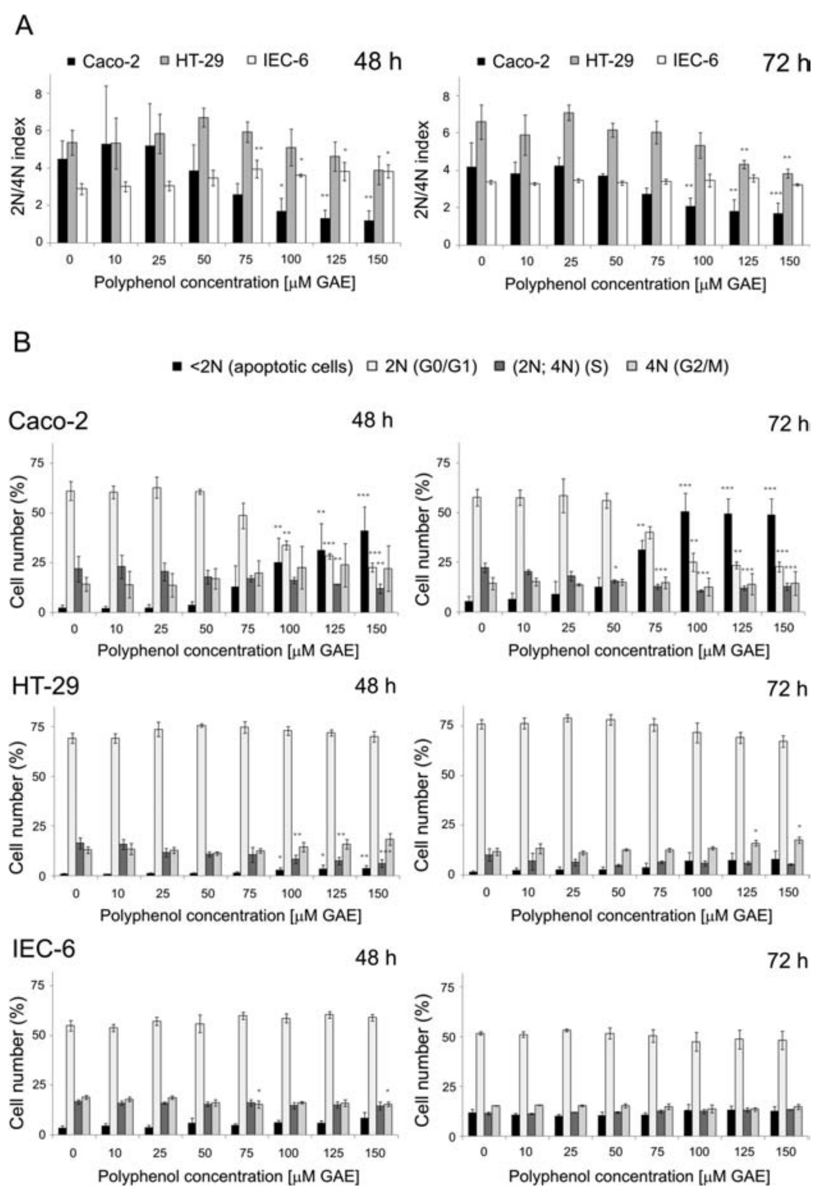

Figure 3. Influence of evening primrose seed polyphenol extract on Caco-2, HT-29, and IEC-6 cell DNA content determined by image cytometry and presented as $2N/4N$ index (A, screening method), followed by cell cycle phase distribution and apoptotic cell analysis by flow cytometry (B, PI staining of DNA). Each value represents the mean value ± SD, $n = 3$ independent experiments. Significance of differences between means: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

tested (100 μM GAE) fraction 2 caused a substantial decrease in fluorescence intensity. Of the four polyphenol extract fractions, fraction 3 had the weakest influence on annexin V binding by IEC-6 within the chosen concentration range.

Influence of Evening Primrose Polyphenols on Cell Membrane Permeability. After 48 and 72 h of incubation with the evening primrose polyphenol extract, cell membrane integrity was assessed on the basis of permeability to a DNA-binding dye, YO-PRO-1, which does not permeate into cells with intact membranes. For Caco-2 a pronounced, statistically significant ($p < 0.001$), and dose-dependent increase in the fluorochrome incorporation was observed after 48 h (Figure 2C). After next 24 h, increased permeability to YO-PRO-1 was detected only at higher concentrations of the extract, whereas at lower ones the permeability generally decreased with reference to control. A marked, statistically significant, and dose-dependent increase in Caco-2 cell permeability was observed for 75 μM GAE flavanol preparation, for polyphenol extract fractions 2 and 3 and, to a lesser extent, for 100 μM GAE fraction 1A (data not shown). An increase in YO-PRO-1 incorporation was also noted for HT-29 and IEC-6 cells after incubation with the extract; however, the effect was not so pronounced as for Caco-2 cells, particularly after 48 h (Figure 2C).

DISCUSSION

We decided to assess the apoptosis-inducing activity of polyphenols from evening primrose seeds in two human colorectal adenocarcinoma cell lines, Caco-2 and HT-29. Caco-2 cells are resistant to some cytostatic drugs and apoptosis-inducing factors,[24] whereas HT-29 cells have a very high level of ARC (an endogenous apoptosis inhibitor) and carry an active Stat6 signaling pathway, as well as a constitutive autophagic pathway,

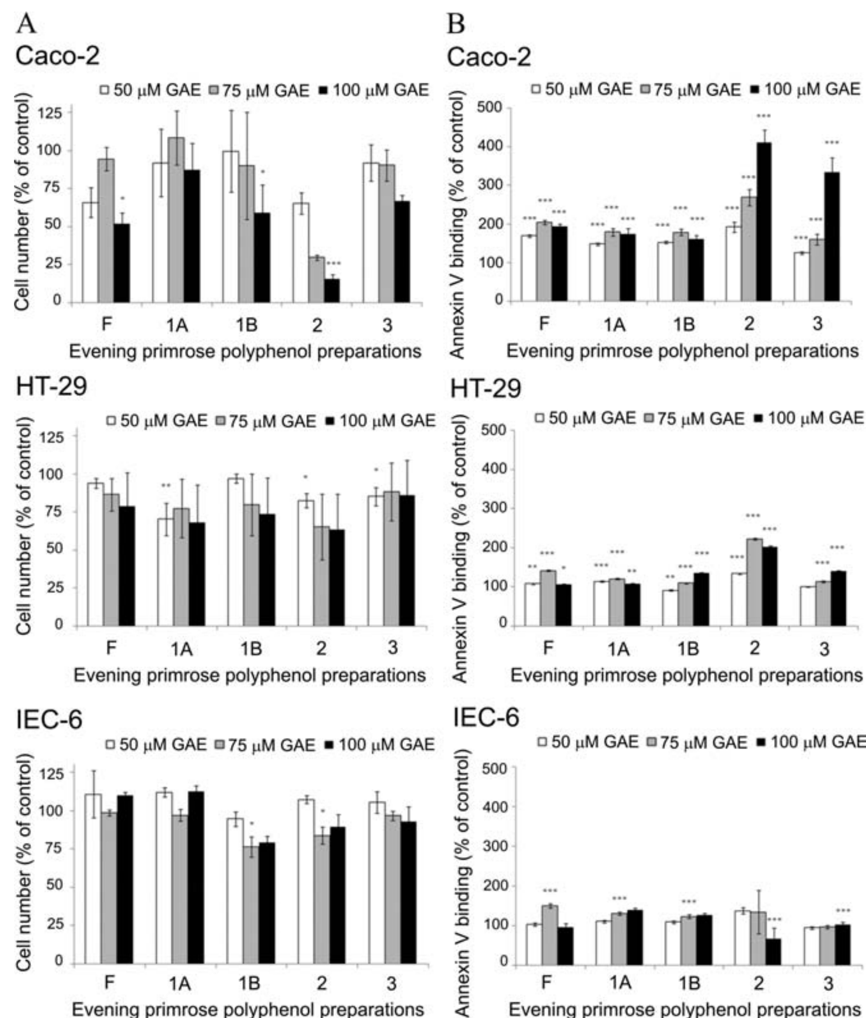

Figure 4. (A) Influence of evening primrose seed polyphenol extract fractions and flavanol preparation on Caco-2, HT-29, and IEC-6 cell number determined by image cytometry after 72 h of incubation, calculated as the sum of Hoechst-33342-stained cell nuclei counted in 16 microscopic fields per well. Each value represents the mean value ± SEM, $n = 3$ independent experiments. Significance of differences between means: ∗, $p < 0.05$, ∗∗, $p < 0.01$, ∗∗∗, $p < 0.001$ versus control. (B) Influence of evening primrose extract fractions on Caco-2, HT-29, and IEC-6 cell annexin V binding determined by image cytometry after 72 h of incubation. Data are expressed as the percentage of control. Each value represents the mean value ± SEM, $n = 400-8500$ Caco-2 cells, $n = 400-8500$ HT-29 cells, $n = 100-1200$ IEC-6 cells. Significance of differences between means: ∗∗∗, $p < 0.001$ versus control.

that altogether enhance their resistance to apoptosis in comparison to Caco-2.[25−27] We also used rat normal intestinal IEC-6 cells in our study to verify whether the proapoptotic activity of the extract is selective toward cancer cells. We tested the polyphenol and flavanol preparations and the polyphenolic fractions obtained according to the procedure presented in Figure 1. The *O. paradoxa* polyphenolic extracts were analyzed by MALDI-TOF MS (Table 3 and mass spectra in Supporting Information).

Our HPLC data show that the polyphenol extract contained mainly hydroxybenzoic acids and flavanols, whereas no hydroxycinnamic acids and flavonols were detected in the preparation by means of this technique (Table 1). Similar results were obtained for the flavanol preparation. High MW flavanols were major constituents of fractions 1A, 1B, and 3 of the polyphenol preparation, whereas hydroxybenzoic acids and low MW flavanols predominated in fraction 2 (Table 2).

The extract inhibited Caco-2 growth by inducing apoptosis in a dose-dependent manner within a concentration range that was estimated to be achievable in the gastrointestinal tract.[7] After 72 h of incubation with the extract at the highest concentration tested, the number of Caco-2 cells decreased to 19% of control, and 48.8% of the cells were identified as apoptotic (vs 5.4% in control), as determined on the basis of cell DNA content measurement by flow cytometry. Under the same conditions only 8% of HT-29 (vs 1.6% in control) and 12.6% of IEC-6 cells (vs 11.8% in control) exhibited hypodiploid DNA content. Thus, after the polyphenol treatment no significant increase in nuclear DNA fragmentation characteristic of apoptosis was detected by flow cytometry of PI-stained HT-29 and IEC-6 cells and by microscopic observations of the cells stained with DAPI. The reduction in HT-29 and IEC-6 cell number (45 and 56% of control, respectively, at 150 μM GAE after 72 h of incubation) may result from inhibition of cell proliferation without phase-specific cell cycle arrest, as we did not observe such an arrest for any of the three types of cells.

The extract fractions were also tested by image cytometry within the concentration range chosen for the extract. The

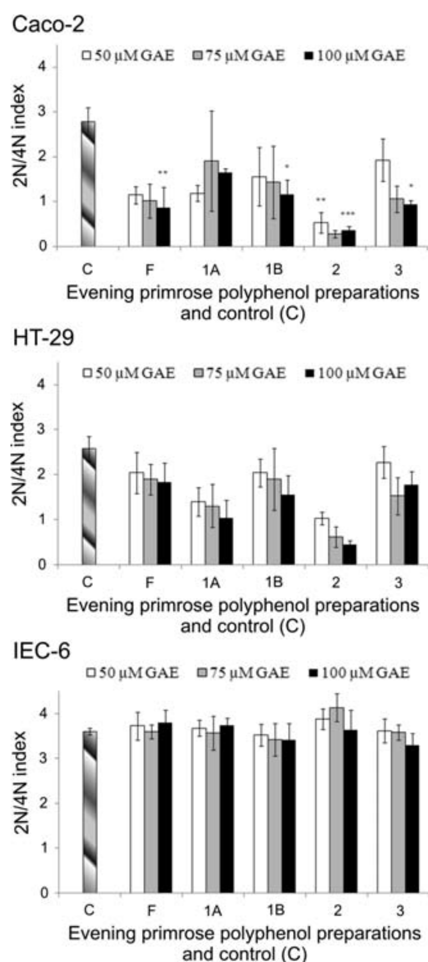

Figure 5. Influence of evening primrose seed polyphenol extract fractions and flavanol preparation on Caco-2, HT-29, and IEC-6 cell DNA content determined by image cytometry after 72 h of incubation and presented as $2N/4N$ index. Each value represents the mean value ± SEM, $n = 3$ independent experiments. Significance of differences between means: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ versus control.

fractions not only strongly and dose-dependently reduced Caco-2 cell number but also had a marked influence on HT-29 growth, while IEC-6 cells were affected to a lesser extent. The stimulation of IEC-6 cell growth by the flavanol preparation at the lowest concentration tested is in line with other results, since proanthocyanidins (flavanols) were demonstrated to enhance normal cell proliferation, as opposed to the effect exerted toward cancer cells.[28] The stimulation of IEC-6 growth only at the lowest concentration tested could be explained as a hormetic effect.[29]

IEC-6 cells showed increased cell permeability to YO-PRO-1, but microscopic observations of the cells treated with polyphenols did not reveal any significant morphological changes versus control (data not shown). Furthermore, the results of flow cytometry and DAPI staining did not indicate that the cells were undergoing apoptosis at the time of harvesting. The results of the annexin V binding assay suggest that IEC-6 cells incubated with evening primrose polyphenols (particularly at higher concentrations) were to some extent more prone to apoptosis than the control cells. However, it should be underscored that apoptotic IEC-6 cells constituted at most 8.4 versus 3.6% in control after 48 h of incubation, and 12.6 versus 11.8% in control after 72 h, which was definitely less than in the case of Caco-2.

In contrast, for Caco-2 the increased permeability to YO-PRO-1 correlated with an increase in annexin V binding, an increase in hypodiploid peak, and noticeable changes in nuclear morphology. The slightly increased cell permeability was also observed for HT-29 and IEC-6 cells treated with the polyphenol extract. However, neither flow cytometry nor DAPI staining revealed significant apoptosis induction in the cells.

Taking into account some data, we postulate that changes in cell membrane permeability to YO-PRO-1 may not be correlated with cell viability. YO-PRO-1 crosses cell membrane in a P2X7-dependent manner and has been used in studies on P2X7 agonists and antagonists.[30] It was demonstrated that approximately 50% of P2X7 receptors present in cell membrane are found within lipid rafts,[31] and polyphenols were demonstrated to interfere with lipid raft organization.[32] As far as we know, there are no literature data on P2X7 receptor expression in IEC-6 cells and in undifferentiated HT-29 cells. We therefore carried out RT-PCR analysis to check whether P2X7 is transcribed in IEC-6, HT-29, and Caco-2 cells used in our study, and the results indicate that P2X7 mRNA is present in all of the cell lines (data not shown).

We suggest that the high permeability of IEC-6 cell membrane to YO-PRO-1 resulted from activation of P2X7 receptors by polyphenols through interference with lipid raft organization. In the present work we did not investigate the influence of polyphenols on the rafts, but our results on high membrane permeability to YO-PRO-1 could be used as a starting point for further studies.

Flavonoids were shown to influence phase transition and lateral segregation of lipids, thus influencing the formation of lipid rafts located in cell membranes.[33] The rafts have recently been recognized as signal processing hubs, and (−)-epigallocatechin gallate (EGCG) was suggested to exert its cancer chemopreventive effects through directly targeting the hubs.[32] The inhibitory effect of EGCG on the activation of EGF receptor and the downstream signaling pathways in colon cancer HT-29 cells was shown to be associated with altered lipid order.[34] Gallates may initiate the membrane rigidifying process and lead to aggregation of some proteins and lipids in clusters.[35] One can suggest that components of gallate-rich evening primrose extract may change lipid raft organization and trigger apoptosis in Caco-2 cells. HT-29 and IEC-6 cells seem to be less susceptible to the proapoptotic activity of the extract, probably due to differences in cell membrane structure/composition. However, the influence of the evening primrose polyphenol extract on cell membranes remains unclear, and additional studies on interactions between their components are necessary to confirm the above hypothesis.

Analysis of the results presented in Figures 4 and 5 leads to the conclusion that fraction 2 is one of the most active among the *O. paradoxa* polyphenol fractions. The low MW polyphenol-enriched fraction 2 caused the strongest decrease in Caco-2 cell number, the most pronounced decrease in Caco-2 and HT-29 DNA content presented as $2N/4N$ index, and the highest increase in Caco-2 and HT-29 annexin V binding. Some components of fraction 2 were tentatively identified by MALDI-TOF MS (Table 3). Among them are supposed to be phenolic acids that have low MW. As ellagic acid and quercetin have MW of 302, the MALDI-TOF MS peak at m/z of 303.1 could represent protonated forms of both compounds. However, quercetin is a

flavonol, and the flavonol content in the polyphenol and flavanol preparations tested in the present work was estimated to be equal to zero on the basis of HPLC analysis. Thus, the peaks at m/z of 301.1, 303.1, and 340.9 presumably represent the $[M - H]^-$, $[M + H]^+$, and $[M + K]^+$ forms, respectively, of ellagic acid. The presence of ellagic acid in fraction 2 was confirmed by HPLC (data not shown). As mentioned above, ellagic acid was identified in the evening primrose (*O. paradoxa*) defatted seed extract in the free form and as a component of an ellagitannin, oenothein B.[1] The acid was reported to induce apoptosis in human colon cancer Caco-2 cells.[8] Another phenolic acid identified in the extract,[1] gallic acid, triggered apoptosis in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines[36] and in human leukemia HL-60 cells.[37] It blocked the growth of DU145 prostate cancer cells at G2/M phase, triggered their apoptotic death, and acted synergistically with doxorubicin in suppressing cell growth.[38] This phenolic acid was identified as one of the major constituents of grape seed extract showing pronounced anticancer activity against human prostate cancer cells both in vitro and in vivo, as it inhibited prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate (TRAMP) model.[39]

In light of the influence of gallates on cell membrane, as outlined above,[32−35] it is worth emphasizing that we tentatively identified in fraction 2 the following compounds: (epi)catechin gallate, hexahydroxydiphenyl (HHDP)-digalloylglucose, B-type dimeric procyanidin digallate and/or B-type dimer of (epi)-catechin gallate, as well as tetra- and pentagalloylglucose (PGG). (−)-Epicatechin gallate and PGG were previously identified in evening primrose (*O. paradoxa*) defatted seed extract,[1] and the seeds were obtained from the same source as in our study.

In contrast to fraction 2, all of the remaining fractions of the polyphenol preparation tested in the present work were enriched with high MW compounds. Their mean DP was above 3 (and was highest for fraction 1B), whereas fraction 2 had a mean DP of 1.7. In the present work we used the total polyphenol content values of the extract expressed as gallic acid. For convenience, we expressed the extract concentrations in μM GAE instead of μg GAE/mL. The discrepancy in the polyphenol content expressed as (+)-catechin, gallic acid, or ellagic acid equivalents results from the fact that the assay involving the Folin−Ciocalteu reagent is based on antioxidant activity,[15] which in turn is structure-dependent.[4,12] It should also be underscored that the extract contains not only low MW polyphenols (such as (epi)catechin and phenolic acids) but also high MW compounds. Besides procyanidins (oligo- and polymers of (epi)catechin with MW of the monomeric unit equal to 290) the evening primrose seeds contain hydrolyzable tannins such as PGG with a MW of 940 and oenothein B with a MW of 1571 (Table 3 and ref 1). Altogether, for the extract fractions enriched with high MW molecules, the molar concentrations of individual constituents were much lower than for fraction 2. As a consequence, although our results indicate that fraction 2 enriched with low MW polyphenols was the most potent in terms of apoptosis induction among all of the extract fractions, the remaining fractions were tested at effectively lower concentrations of individual constituents than in the case of fraction 2. We therefore suggest that the constituents of not only fraction 2 but also the remaining fractions account for the proapoptotic potential of the evening primrose seed extract toward gastrointestinal tract derived Caco-2 cancer cells.

Fraction 2 exhibited the highest antioxidative efficiency in our study (Table 2S); the remaining fractions had similar mean DP values (Table 2) but differed in TEAC values and in the extent of inhibition of linoleic acid oxidation (Table 2S). In contrast to the systemic level, where concentrations of individual phenolics rarely exceed 1 μM, polyphenol concentrations in the gastrointestinal tract were estimated to reach the low millimolar range.[7] Millimolar concentrations are likely to be sufficient for antioxidant activity; however, according to Holst and Williamson[40] defense against hydroxyl radicals by small molecules (such as low MW polyphenols) requires concentrations in the high millimolar range. Therefore, we presume that the concentrations chosen for our study would be too low for efficient antioxidant activity in the gastrointestinal tract.

Finally, it is worth pointing out that phenolic compounds strongly interfere with some assays used to evaluate their influence on cell growth or viability, particularly with those based on tetrazolium salt reduction to formazan such as MTT and MTS assays.[41,42] We therefore decided to determine cell number by image cytometry of Hoechst-stained cells. The main advantages of this method are high accuracy and lack of interference by polyphenols.

In conclusion, we demonstrated for the first time a dose-dependent induction of apoptosis in human colorectal adenocarcinoma Caco-2 cells by evening primrose (*O. paradoxa*) waste seed extract within a concentration range that was estimated to be achievable in the gastrointestinal tract.[7] The reduction of human colorectal adenocarcinoma HT-29 and rat normal intestinal IEC-6 cell number (45 and 56% of control, respectively) may result from inhibition of cell proliferation without phase-specific cell cycle arrest, as such an arrest was not observed for any of the three types of cells in our study. The majority of constituents of evening primrose waste seed extract identified to date may contribute to its proapoptotic activity toward Caco-2 cells.

■ ASSOCIATED CONTENT

S Supporting Information. Additional figures and tables. Figure 1S-A: MALDI-TOF mass spectrum of evening primrose seed polyphenol extract recorded in reflection mode (positive ion detection). Figure 1S-B: MALDI-TOF mass spectrum of evening primrose seed polyphenol extract recorded in reflection mode (negative ion detection). Figure 1S-C: MALDI-TOF mass spectrum of fraction 2 of the evening primrose seed polyphenol extract recorded in reflection mode (positive ion detection). Figure 1S-D: MALDI-TOF mass spectrum of fraction 2 of the evening primrose seed polyphenol extract recorded in reflection mode (negative ion detection). For all parts of Figure 1S, the numbers from 1 to 24 refer to compounds listed in Table 3. Figure 2S: Representative histograms of flow cytometry cellular DNA content analysis of Caco-2, HT-29, and IEC-6 cells, untreated (C) and treated with 150 μM GAE evening primrose polyphenol preparation (PP). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: + 48 42 631 34 97. Fax: + 48 42 636 66 18. E-mail: sylwia.gorlach@gmail.com.

Author Contributions

[‖]These authors contributed equally to the work.

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■ ABBREVIATIONS USED

$ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; DAPI, 4′,6-diamidino-2-phenylindole; DHB, 2,5-dihydroxybenzoic acid; DP, degree of polymerization; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; HCS, high content screening; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; m/z, mass-to-charge ratio; PGG, pentagalloylglucose; PI, propidium iodide; TEAC, Trolox equivalent antioxidant capacity; TIP, tentatively identified peak.

■ REFERENCES

(1) Kiss, A. K.; Derwińska, M.; Dawidowska, A.; Naruszewicz, M. Novel biological properties of *Oenothera paradoxa* defatted seed extracts: effects on metallopeptidase activity. *J. Agric. Food Chem.* **2008**, *56*, 7845–7852.

(2) Arimura, T.; Kojima-Yuasa, A.; Tatsumi, Y.; Kennedy, D. O.; Matsui-Yuasa, I. Involvement of polyamines in evening primrose extract-induced apoptosis in Ehrlich ascites tumor cells. *Amino Acids* **2005**, *28*, 21–27.

(3) Pellegrina, C. D.; Padovani, G.; Mainente, F.; Zoccatelli, G.; Bissoli, G.; Mosconi, S.; Veneri, G.; Peruffo, A.; Andrighetto, G.; Rizzi, C.; Chingola, R. Anti-tumour potential of a gallic acid-containing phenolic fraction from *Oenothera biennis*. *Cancer Lett.* **2005**, *226*, 17–25.

(4) Jaszewska, E.; Kośmider, A.; Kiss, A. K.; Naruszewicz, M. Pro-oxidative and pro-apoptotic action of defatted seeds of *Oenothera paradoxa* on human skin melanoma cells. *J. Agric. Food Chem.* **2009**, *57*, 8282–8289.

(5) Bałasińska, B. Hypocholesterolemic effect of dietary evening primrose (*Oenothera paradoxa*) cake extract in rats. *Food Chem.* **1998**, *63*, 453–459.

(6) Serrano, J.; Puupponen-Pimi, R.; Dauer, A.; Aura, A. M.; Saura-Calixto, F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Mol. Nutr. Food Res.* **2009**, *53* (Suppl. 2), 310–329.

(7) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130* (Suppl. 8), 2073–2085.

(8) Larrosa, M.; Tomás-Barberán, F. A.; Espin, J. C. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J. Nutr. Biochem.* **2006**, *17*, 611–625.

(9) Cerdá, B.; Cerón, J. J.; Tomás-Barberán, F. A.; Espín, J. C. Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. *J. Agric. Food Chem.* **2003**, *51*, 3493–3501.

(10) González-Sarrías, A.; Espín, J. C.; Tomás-Barberán, F. A.; García-Conesa, M. T. Gene expression, cell cycle arrest and MAPK signalling regulation in Caco-2 cells exposed to ellagic acid and its metabolites, urolithins. *Mol. Nutr. Food Res.* **2009**, *53*, 686–698.

(11) Yamakoshi, J.; Saito, M.; Kataoka, S.; Tokutake, S. Procyanidin-rich extract from grape seeds prevents cataract formation in hereditary cataractous (ICR/f) rats. *J. Agric. Food Chem.* **2002**, *50*, 4983–4988.

(12) Cerdá, B.; Espín, J. C.; Parra, S.; Martínez, P.; Tomás-Barberán, F. A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolised into bioavailable but poor antioxidant hydroxy-6*H*-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220.

(13) Lee, J. H.; Johnson, J. V.; Talcott, S. T. Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. *J. Agric. Food Chem.* **2005**, *53*, 6003–6010.

(14) Oszmianski, J. Sposob otrzymywania aktywnych biologicznie oligomerow proantocyjanidyn z surowcow roslinnych (Method of preparation of biologically active oligomeric proanthocyanidins from plant raw materials). Polish Patent P-296521, 1992.

(15) Peri, C.; Pompei, G. An assay of different phenolic fractions in wines. *Am. J. Enol. Vitic.* **1971**, *22*, 55–57.

(16) Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica* II. *J. Sci. Food Agric.* **1959**, *10*, 63–68.

(17) Rösch, D.; Bergmann, M.; Knorr, D.; Kroh, L. W. Structure—antioxidant efficiency relationships of phenolic compounds and their contribution to the antioxidant activity of sea buckthorn juice. *J. Agric. Food Chem.* **2003**, *51*, 4233–4239.

(18) Hartzfeld, P. W.; Forkner, R.; Hunter, M. D.; Hagerman, A. E. Determination of hydrolyzable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. *J. Agric. Food Chem.* **2002**, *50*, 1785–1790.

(19) Oszmianski, J.; Bourzeix, M. Comparison of methods for determining the content and polymerization of proanthocyanidins and catechins. *Pol. J. Food Nutr. Sci.* **1996**, *46*, 43–50.

(20) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radicals Biol. Med.* **1999**, *26*, 1231–1237.

(21) Benzie, J. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

(22) Szwajgier, D.; Targoński, Z. Comparison of in vitro activities of malt, hops, worts and lager type beer. *Pol. J. Food Nutr. Sci.* **2000**, *50*, 53–59.

(23) Dyrby, M.; Westergaard, N.; Stapelfeldt, H. Light and heat sensitivity of red cabbage extract in soft drink model systems. *Food Chem.* **2001**, *72*, 431–437.

(24) Cucina, A.; Biava, P. M.; D'Anselmi, F.; Coluccia, P.; Conti, F.; di Clemente, R.; Miccheli, A.; Frati, L.; Gulino, A.; Bizzarri, M. Zebrafish embryo proteins induce apoptosis in human colon cancer cells (Caco2). *Apoptosis* **2006**, *11*, 1617–1628.

(25) Mercier, I.; Vuolo, M.; Jasmin, J. F.; Medina, C. M.; Williams, M.; Mariadason, J. M.; Qian, H.; Xue, X.; Pestell, R. G.; Lisanti, M. P.; Kitsis, R. N. ARC (apoptosis repressor with caspase recruitment domain) is a novel marker of human colon cancer. *Cell Cycle* **2008**, *7*, 1640–1647.

(26) Li, B. H.; Yang, X. Z.; Li, P. D.; Yuan, Q.; Liu, X. H.; Yuan, J.; Zhang, W. J. IL-4/Stat6 activities correlate with apoptosis and metastasis in colon cancer cells. *Biochem. Biophys. Res. Commun.* **2008**, *369*, 554–560.

(27) Bauvy, C.; Gane, P.; Arico, S.; Codogno, P.; Ogier-Denis, E. Autophagy delays sulindac sulfide-induced apoptosis in the human intestinal colon cancer cell line HT-29. *Exp. Cell Res.* **2001**, *268*, 139–149.

(28) Bagchi, D.; Bagchi, M.; Stohs, S.; Ray, S. D.; Sen, C. K.; Preuss, H. G. Cellular protection with proanthocyanidins derived from grape seeds. *Ann. N.Y. Acad. Sci.* **2002**, *957*, 260–270.

(29) Mattson, M. P. Hormesis defined. *Ageing Res. Rev.* **2008**, *7*, 1–7.

(30) Dutot, M.; Liang, H.; Pauloin, T.; Brignole-Baudouin, F.; Baudouin, C.; Warnet, J. M.; Rat, P. Effects of toxic cellular stresses and divalent cations on the human P2X7 cell death receptor. *Mol. Vis.* **2008**, *14*, 889–897.

(31) Gonnord, P.; Delarasse, C.; Auger, R.; Benihoud, K.; Prigent, M.; Cuif, M. H.; Lamaze, C.; Kanellopoulos, J. M. Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. *FASEB J.* **2009**, *23*, 795–805.

(32) Patra, S. K.; Rizzi, F.; Silva, A.; Rugina, D. O.; Bettuzzi, S. Molecular targets of (−)-epigallocatechin-3-gallate (EGCG): specificity and interaction with membrane lipid rafts. *J. Physiol. Pharmacol.* **2008**, *59* (Suppl. 9), 217–235.

(33) Tarahovsky, Y. S.; Muzafarov, E. N.; Kim, Y. A. Rafts making and rafts braking: how plant flavonoids may control membrane heterogeneity. *Mol. Cell. Biochem.* **2008**, *314*, 65–71.

(34) Adachi, S.; Nagao, T.; Ingolfsson, H. I.; Maxfield, F. R.; Andersen, O. S.; Kopelovich, L.; Weinstein, I. B. The inhibitory effect of (−)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. *Cancer Res.* **2007**, *67*, 6493–6501.

(35) Tarahovsky, Y. S. Plant polyphenols in cell−cell interaction and communication. *Plant Signal. Behav.* **2008**, *3*, 609–611.

(36) Yoshioka, K.; Kataoka, T.; Hayashi, T.; Hasegawa, M.; Ishi, Y.; Hibasami, H. Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncol. Rep.* **2000**, *7*, 1221–1223.

(37) Okuda, T. Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry* **2005**, *66*, 2012–2031.

(38) Chen, H. M.; Wu, Y. C.; Chia, Y. C.; Chang, F. R.; Hsu, H. K.; Hsieh, Y. C.; Chen, C. C.; Yuan, S. S. Gallic acid, a major component of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells. *Cancer Lett.* **2009**, *286*, 161–171.

(39) Raina, K.; Rajamanickam, S.; Deep, G.; Singh, M.; Agarwal, R.; Agarwal, C. Chemopreventive effects of oral gallic acid feeding on tumor growth and progression in TRAMP mice. *Mol. Cancer Ther.* **2008**, *7*, 1258–1267.

(40) Holst, B.; Williamson, G. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. *Curr. Opin. Biotechnol.* **2008**, *19*, 73–82.

(41) Bernhard, D.; Schwaiger, W.; Crazzolara, R.; Tinhofer, I.; Kofler, R.; Csordas, A. Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. *Cancer Lett.* **2003**, *195*, 193–199.

(42) Talorete, T. P.; Bouaziz, M.; Sayadi, S.; Isoda, H. Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells. *Cytotechnology* **2006**, *52*, 189–198.