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ARTICLE

Anticancer Activity of Rhamnoallosan against DU-145 Cells Is Kinetically Complementary to Coexisting Polyphenolics in *Psidium guajava* Budding Leaves

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Psidium guajava L. is a valuable farm fruit plant having many medicinal uses. Previously its budding leaves (PE) were shown to contain huge amounts of soluble polyphenolics (SP) including (in mg/g) gallic acid (348), catechin (102), epicatechin (60), rutin (100), quercetin (102), and rutin (100) and to exhibit potent anticancer activity. However, reconstitution of these polyphenolics recovered only 40% of the original bioactivity, and the soluble carbohydrate (SC) portion in PE was suspected to contribute the remaining. PE contained a novel rhamnoallosan, which had a carbohydrate/protein (w/w) ratio = 29.06%/10.27% (=2.83, average molecular mass of 5029 kDa), characteristically evidencing a peptidoglycan, consisting of a composition (mole % ratio) of rhamnose/allose/arabinose/tallose/xylose/fucose/glucose/mannose/galactose = 36.05:24.24:8.76:7.95:7.37:5.90:3.69:3.19:2.85 and of amino acid (in wt %) glycine/leucine/proline/alanine/methionine/isoleucine/valine/histidine/tyrosine/phenylalanine/cysteine/aspartic acid/lysine/glutamic acid = 37.12:12.68:10.05:8.97:5.99:4.89:4.83:4.25:4.05:2.78:1.86:1.10:0.73:0.70. Kinetic analysis showed comparable apparent cell-killing rate coefficients (k_{app}) to be 4.03×10^3 and 2.92×10^3 cells mg^{-1} h^{-1}, respectively, by SP and SC, evidencing the complementary anti-DU-145 bioactivity in nature.

KEYWORDS: Rhamnoallosan peptidoglycans; rhamnose; allose; prostate cancer; *Psidium guajava* budding leaves

INTRODUCTION

Psidium guajava L. (guava) has been traditionally used as a valuable folkloric herbal medicine for the treatment of a diversity of diseases. In China, India, and Taiwan, extracts of roots, barks, and leaves are used to treat many bacterial and viral infections, diarrhea, dysentery, and stomach upsets (*1*). Even today, guava leaves are still cited in the Dutch Pharmacopeia (*1*). In addition, guava leaves can be potentially good as preventive therapeutics for many cardiovascular (*2*) and neural degenerative (*3*) diseases. Up to the present, 60 compounds in essential oils of the guava leaves have been identified (*1*, *4*), among which β-sitosterol glucoside and brahmic acid are the two main hypoglycemic constituents (*1*, *5*). In addition, aqueous extracts of *P. guajava* leaves (PE) and barks are effective central nervous system depressants, having useful cough suppressant, hypotensive (lowering blood pressure), sedative, and analgesic (pain reliever) qualities. All of these effects have been ascribed to its extraordinary free radical scavenging and antioxidative capabilities exerted by the huge contents of total polyphenolics, flavonoids, and terpenoids in PE (*6*). The flavonoids are suggestive of excellent antiglycative agents (*1*,*6*). For example, rutin is capable of inhibiting early glycation products (*1*,*7*). Much of the literature has pointed out that the oxidative stress induced by glycation in reality plays an important role in diabetic pathology (*1*,*8*). Long-term hyperglycemia may elicit glucose autoxidation, protein glycation, and advanced glycation end product (AGEs) production. Moreover, ellagic acid is also known for its astringent activity beneficial against diarrhea and dysentery (*1*, *9*). Currently, PE has been identified to be effective against prostate cancer LNCaP (unpublished data, courtesy of Dr. Chen, 2009) and DU-145 cells (*10*). PE successfully down-regulated the expression of MMP-2 and MMP-9 in these cancer cells. Pronounced overexpression of MMPs is required for both tumor invasion and tumor angiogenesis in foreign tissues (*1*, *11*).

Recently, our laboratory has identified seven major polyphenolics in PE including catechin (132 mg/g), epicatechin (60 mg/g), gallic acid (86 mg/g), quercetin (56 mg/g), rutin (110 mg/g), naringenin (trace), and kaempferol (trace) (*12*). Astonishingly, on reconstitution of these total phenolic acids, only 40% of the bioactivity originally found in total PE was recovered (Appendix **Figure A1**), implicating alternate coexisting bioactive components

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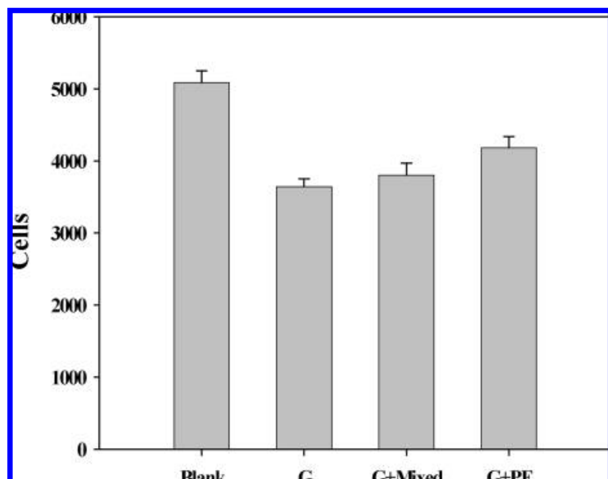

Figure A1. Effect of mixed polyphenolics and PE on cell viability of HUVEC induced by glucose (courtesy of Professor C.-L. Hseih, 2008, unpublished data). HUVEC was incubated with G (30 mM) at 37 °C for 2 days in the presence and absence of GA, C, EC, R, and Q (gallic acid, catechin, epicatechin, rutin, and quercetin, 50 μM, respectively). Blank, without the addition of glucose or any polyphenolic compound; G, glucose; G+Mixed, glucose plus mixed polyphenolics (GA, C, EC, R. and Q). Data are expressed as mean ± SD from triplicate experiments. ∗, $p < 0.05$ compared with blank; #, $p < 0.05$ compared with control (G). As can be seen, the mixed polyphenolics revealed only around 40% of bioactivity as exhibited by PE assumed to have a bioactivity of 100%.

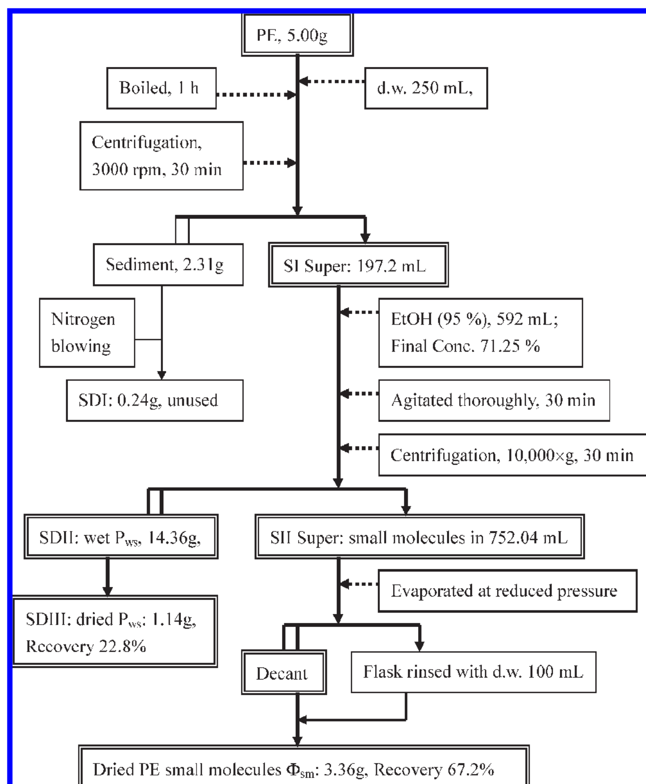

Figure 1. Flowchart for the isolation of soluble polysaccharides and small molecular weight phenolics from PE.

in PE. Literally, a diversity of polysaccharides isolated from mushroom have been identified to be excellent biological response modifiers in enhancing a number of biological functions (*13*). We explored a unique novel peptidoglycan present in PE in this study and demonstrated its antiprostate DU-145 cancer bioactivity to be complementary to that of the polyphenolics present in PE.

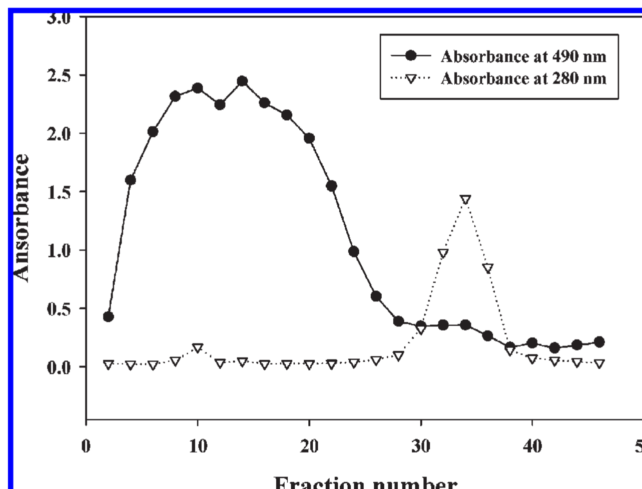

Figure 2. Isolated soluble polysaccharide fractions of PE analyzed by gel permeation chromatography measured at 490 and 280 nm, respectively. The optical density measured at 280 nm represents the protein content, and that measured at 490 nm resulting from phenol−sulfuric acid stands for the content of polysaccharides. The average molecular mass of water-soluble polysaccharides in PE was 5029 kDa.

Table 1. Monosaccharide and Amino Acid Compositions of the Water-Soluble Polysaccharide Fraction (P_{ws}) Isolated from *Psidium guajava* L. Budding Leaf Extract[a]

monosaccharide	mol %	monosaccharide	mol %
rhamnose	36.05	fucose	5.90
allose	24.24	glucose	3.69
arabinose	8.76	mannose	3.19
tallose	7.95	galactose	2.85
xylose	7.37		
amino acid	**wt %**	**amino acid**	**wt %**
glycine	37.12	histidine	4.25
leucine	12.68	tyrosine	4.05
proline	10.05	phenylalanine	2.78
alanine	8.97	cysteine	1.86
methionine	5.99	aspartic acid	1.10
isoleucine	4.89	lysine	0.73
valine	4.83	glutamic acid	0.70

[a] The carbohydrate and protein contents were, respectively, 29.06 and 10.27% w/w in P_{ws}, giving a ratio of 2.83 (w/w), characteristically indicating in nature a peptidoglycan. The average molecular mass of P_{ws} was 5029 kDa.

MATERIALS AND METHODS

Chemicals and Reagent. The lyophilized aqueous extract of *P. guajava* L. budding leaves (PE) was a gift from Dr. Hsieh (Laboratory of Molecular Nutraceutics, Department of Food and Nutrition, Hungkuang University, Taichung, Taiwan). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Langley, OK). All other chemicals used in this study were purchased from authentic sources and of the highest grade and purity.

Cell Lines. As previously cited (*10*), virally transformed normal human prostate epithelial PZ-HPV-7 cells and human carcinoma DU-145 cells we purchased from the Culture Collection and Research Center (CCRC) of the Food Industry Research and Development Institute (FIRDI) (Hsinchu, Taiwan, ROC). DU-145 cells were cultured in RPMI 1640 medium with 5% FBS and 1% penicillin−streptomycin cocktail (Cellgro, Mediatech Inc., Herndon, VA) at 37 °C in a humidified

Table 2. Comparison of the Monosaccharide Composition in the Aqueous Extract of *Psidium guajava* L. Budding Leaves with the Cited Polysaccharides

authors	source	monosaccharide composition/mol % ratio	reference
Chen et al. (2009)	aqueous extract of *P. guajava* L. budding leaves	Rha:All:Ara:Tal:Xyl:Fuc:Glc:Man:Gal (mol %) = 36.05:24.24:8.76:7.95:7.37:5.90:3.69:3.19:2.85	this paper
Fischer et al. (2001)	green Arabica and Robusta coffee beans	Rha:Fuc:Ara:Xyl:Man:Gal:Glc (mol %) = 0.3:0.4:10.8:0.6:44.8:25.5:17.6	(*24*)
Oosterveld et al. (2003)	*Coffea arabica* beans	Rha:Ara:Xyl:Man:Gal:Glc:UA (mol %) = 2:13:0:39:38:1:7	(*25*)
Kurz et al. (2008)	apricots, peaches and pumpkins	Rha:Fuc:Rib:Ara:Xyl:Man:Gal:Glc (wt %) = 5.3:2.0:nd:43.5:16. 0:4.8:19.7:8.7 or 4.2:2.7:nd:46.6:9.6:2.6:27.1:7.6 or 9.7:2.2:nd:7.6:16.2:10.0:13.5:40.8	(*26*)
Lisboa et al. (2005)	*Eucalyptus globulus* kraft black liquors	Rha:Fuc:Ara:Xyl:Man:Gal:Glc (wt %) = 0.5:0.0:1.1:82.5:0.9:4.8:10.2	(*27*)
Chen et al. (2008)	*Ganoderma atrum* fruiting body	Glc:Man:Gal (wt %) = 68.3:13.9:17.8	(*28*)
Lo et al. (2006)	*Lentinula edodes*	Ara:Xyl:Man:Gal:Glc:Rha:Fuc (mol ratio) = 0.79:0.75:3.46:0.36:1.62:0.02:0.04	(*29*)
Sun et al. (2006)	leaf cell wall of forage chicory	Ara:Xyl:Man:Gal:Glc:Rha:Fuc (mg/g) =34.9:23.7:62.7:218.0:1.3:3.6:51.8	(*30*)
Harris et al. (1997)	monocotyledon cell walls	Ara:Xyl:Man:Gal:Glc:Rha:Fuc (wt %) = 5.9:15.1:2.5:56.5:8.7:9.2:2.1	(*31*)
Doco et al. (2001)	wine polysaccharide	Ara:Xyl:Man:Gal:Glc:Rha:Fuc (mg/L) = 45:5:165:125:49 :31:6	(*32*)
Miyajima et al. (2001)	marine sediments	Ara:Xyl:Man:Gal:Glc:Rha:Fuc (μmol/g) = 0.37:0.71:0.58:0.98:0.66:0.52:0.61	(*33*)
Koh et al. (2002)	strawberry cortical and pith tissues	Rha:Fuc:Ara:Xyl:Man:Gal:Glc (mol %) = 8.7:2.3:41.9:9.9:3.5:21.6:12.2	(*34*)
Mayworm et al. (2000)	Vochysiaceae seed cell walls	Rha:Fuc:Ara:Xyl:Man (% w/w) = 4.9:39.6:18.3:7.7:29.4	(*35*)
Reid et al. (1999)	apple suspension cultures	Rha:Fuc:Ara:Xyl:Man:Gal:Glc (% w/w) = 4:2:23:12:2:44:13	(*36*)
Wang et al. (2003)	Chinese *Lingustrum lucidum* polysaccharide	Fuc:Glc:Ara:Rha (mol %) = 1.8:4.58:2.55:1.91	(*37*)
Osborn et al. (1999)	root mucilage of maize	Rha:Fuc:Xyl:Ara:Gal:Glc:Man (mol %) = 0:7.1:2.4:4.7:5.0:79.9:1.0	(*38*)
Wang et al. (2004)	fruiting body of *Hericium erinaceus* Pers	Glc:Gal:Fuc (mol) = 1:2.11:0.423	(*39*)
Park et al. (2004)	*Phellodendron chinesis* Schneid polysaccharide	GlcNAC:Gal:Man:Glc (mol %) = 4.1:1.0:2.6:0.6	(*40*)
Mizuno et al. (1986)	maitake, *Grifola frondosa* polysaccharide	Rha:Fuc:Xyl:Man:Gal:Glc (mol %) = 7.3:12.8:0:8.9:14.2:100	(*41*)
Carnachan et al. (2000)	primary cell walls of the palms *Phoenix canariensis* and *Rhopalostylis sapida*	Rha:Fuc:Ara:Xyl:Man:Gal:Glc (mol %) = 5.6:1.4:54.6:13.3:1.4:19.9:3.8	(*42*)
Lee et al. (2002)	*Antrodia camphorate* polysaccharide	*myo*-inositol:sorbirtol:Fuc:GalN:GlcN:Gal:Glc:Man (μmol/g) = 4.2:0.68:3.24:10.5:26.64:117.49:146.74:69.15	(*43*)

atmosphere of 5% CO_2. The PZ-HPV-7 cells were cultured in keratinocyte serum-free medium supplemented with 5 ng/mL human recombinant EGF and 0.05 mg/mL bovine pituitary extract (Gibco).

Soluble Polysaccharide and Polyphenolics. The water-soluble polysaccharide fraction was isolated according to a similar method previously reported (*14*) with some modifications. Briefly, to 5.00 g of PE was added 250 mL of deionized water. The mixture was boiled for 1 h and centrifuged at 3000 rpm for 30 min; the sediment (2.31 g) was dried under nitrogen air flow to yield a residue of 0.24 g (SDI: recovery = 4.80%). The supernatant (SI Super: 197.2 mL) was added with ethanol (95% v/v, 592 mL) to adjust the ethanol content to a final value of 71.25%. The mixture was agitated for 30 min and centrifuged at 10000*g* for 30 min. The sediment (SDII: wet weight = 14.36 g) was lyophilized to give the water-soluble polysaccharide fraction (SDIII P_{ws}: dried weight = 1.14 g, recovery = 22.80%). The supernatant containing small molecular weight polyphenolics (SII Super, 752.04 mL) was evaporated under reduced pressure and decanted from the evaporation flask (Decant, original concentrate). The empty flask was rinsed with deionized water (dw, 100 mL). The washing was combined with the decant and subjected to lyophilization, yielding 3.36 g of dried fraction of PE, which actually represented the small molecular weight polyphenolics of PE (Φ_{sm}: 3.36 g, recovery = 67.2%) (**Figure 1**).

Molecular Weight Determination. Sample P_{ws} (10 mg) was accurately weighed and placed in a reaction vessel. One milliliter of NaOH (1 N) was added. The mixture was heated in a water bath until dissolved. Two milliliters of deionized water was added. The mixture was agitated for 10 min and filtered through a 0.45 μm micropore to obtain the alkali dissolvable polysaccharides (ADP). An aliquot of 0.5 mL of ADP was injected into a gel permeation chromatography (GPC) column and eluted as directed by Ker et al. (*14*). A total of 50 tubes were collected. The optical density was measured in parallel at 490 and 280 nm, respectively.

Determination of Polysaccharide. To 0.5 mL of ADP was added 0.5 mL of phenol color reagent (5%). The mixture was vigorously agitated to facilitate a homogeneous solution. Sulfuric acid (2.5 mL) was rapidly dropped in. The mixture was agitated thoroughly to facilitate color reaction (orange color). Optical density was measured at 490 nm.

GC-MS Analysis of Polysaccharide Composition: Hydrolysis, Reduction, Derivatization of Monosaccharides. The method of Ker et al. (*14*) was followed. Sample P_{ws} (2 mg) was accurately weighed and

Table 3. Polyphenolic Content and Composition in *Psidium guajava* L. Budding Leaf Aqueous Extract (PE) Identified by LC-ESI/MS[a]

compound	content (mg/g)	retention time (min)	UV λ_{max}	$[M - H]^-$ *m/z*
total polyphenolics (mg of GAE/g of PE)	470.0 ± 48.8			
total flavonoids (mg of CE/g of PE)	248.6 ± 34.2			
gallic acid	348	5.17	270, 225	168.9
catechin	102	31.97	230, 229	289.1
epicatechin	60	36.77	230, 279	289.0
rutin	100	40.23	256	354.0
quercetin	102	51,32	254, 370	301.1
naringenin	minute	55.22	231, 288	271.1
kaempferol	minute	56.39	265,367	285.1

[a] Selective ion monitoring of the $[M - H]^-$ in extracts of guava budding leaves (PE).

transferred into a reaction vessel, to which 2 mL of 2 M trifluoroacetic acid containing 50 μg of deoxyribose as internal standard was added. The mixture was heated with a Cole-Parmer heater at 120 °C for 24 h to facilitate reaction. The remaining procedures were similarly conducted as cited by Ker et al. (*14*). Finally, the CH_2Cl_2 layer was sucked out and dehydrated with anhydrous sodium sulfate. The dehydrated product was transferred into a 1 mL reaction vessel, lyophilized, and analyzed with GC-MS.

Protein Analysis. *Pretreatment*. An amount of 5.1 mg of P_{ws} was accurately weighed and added to a small amount of NaOH (1 N), agitated with a magnetic stirrer to dissolve the solid residue. The solution was made to a volume of 5 mL. Ten microliters of each standard and sample solution was pipetted into a 96-well microplate, to which 200 μL of diluted dye reagent was added to each well. The mixtures were gently votexed and then incubated at ambient temperature for 5 min; the optical density was measured at 595 nm by an ELISA Reader. Authentic bovine serum albumin (BSA) was used to establish the calibration curve, which was used for calculation of the amount of proteins present.

Acid Hydrolysis. Sample P_{ws} (3 g) was accurately weighed and transferred into a 2 mL reaction vessel, to which 2 mL of 6 M HCl was added. After nitrogen air flow for 10 min to drive off oxygen, the vessel was sealed and placed onto a derivatization heater and heated at 110 °C for 24 h until completely hydrolyzed. The mixed amino acid product was lyophilized. The desiccated product was dissolved in 0.3 mL of HCl (0.01 M) to obtain sample solution of a hydrolyzed amino acid mixture (AM).

Derivatization of Amino Acid and Extraction. Standard amino acid solution (0.3 mL of authentic sample solution) and 0.6 mL of AM were respectively placed into 3 mL reaction vessels to which 0.01 mL of internal standard norleucine solution (10 mg/mL) was then added. The mixture was vigorously agitated, and 0.1 mL of ethyl chloroformate was added. After thorough shaking, 1 mL of alcohol−pyridine was added. On addition of 2 mL of chloroform, the mixture was well agitated for 1 min to facilitate derivatization and extraction. The reaction mixture was added to 0.7 mL of water, shaken well, and left to stand for 5 min to ease phase separation. The supernatant was discarded, and the lower layer (i.e., chloroform layer) was transferred into another tube and dehydrated with a sufficient amount of anhydrous sodium sulfate. The dehydrated chloroform extract was transferred into a sample vessel for GC-MS analysis.

GC-MS Operating Conditions. The GC-MS, an FID type gas chromatograph (Agilent 6890, Wilmington, DE) equipped with a HP-5MS column (l = 30 m, i.d. = 0.25 mm, film thickness = 0.25 μm), was used. The mobile phase nitrogen was run at a flow rate of 0.8 mL/min. The detector (FID) and the injection port were maintained at temperature 305 and 300 °C, respectively. The elution was temperature programmed, starting at 50 °C for 1 min, then programmed with at an elevation rate of 10 °C/min to 300 °C and held there for 6.5 min.

Total Polyphenolics. The desiccated herbal aqueous extract (5 mg) was dissolved in an acidic mixed solvent of methanol/water (60: 40, 0.3% HCl). The remaining procedures were conducted according to the method of Hsieh et al. (*12*). The amount of polyphenolics present in the extracts was expressed as milligrams of gallic acid equivalents per gram of sample (mg of GAE/g).

Table 4. Free Radical Scavenging (FRSC) and Ferrous Ion Chelating (FICC) Capabilities of PE[a]

chemical (mg/mL)	FRSC (%)	FICC (%)	AGEs fluorescence (inhibition %)	TBARs (inhibition %)
control			215.3 ± 2.4	0.0 ± 0.0
PE				
0.01	51.7 ± 3.3		78.7 ± 4.3	
0.1	57.4 ± 4.6			
0.5				75.8 ± 2.9
P_{ws}				
0.1	56.3 ± 4.5	90.2 ± 6.7		
0.5	66.4 ± 5.7	91.0 ± 5.2		
Φ_{sm}				
0.1				
0.5				
gallic acid[b]				
0.1	98.1 ± 4.6			
0.5	98.9 ± 6.4			
BHA[b]				
0.1	93 ± 5.8			
0.5	96 ± 6.4			
citric acid[b]				
0.1		39.2 ± 3.7		
0.5		40.2 ± 5.2		
AG[b], 0.01	positive control	positive control	194.6 ± 9.2	68.0 ± 2.2

[a] The majority of data were courtesy of Dr. C.-L. Hsieh. All figures state data expressed as mean ± SD obtained from triplicate experiments. Significance level ($p < 0.05$). [b] Gallic acid and BHA were used as references for determination of FRSC; citric acid was used as reference of FICC; aminoguanidine (AG) was used as the control of FRSC, FIRC, AGEs and TBARs.

Total Flavonoids. The method described by Hsieh et al. (*6*) was followed. Briefly, to 1 mL of the aqueous solution of herbal extract (1 mg/mL) was added 1.25 mL of deionized water and then mixed thoroughly with 75 μL of $NaNO_2$ (5%). The remaining procedures were conducted in the same manner as described. The absorbance was measured at 510 nm and calculated against the calibration curve established using catechin as the reference standard and expressed as milligrams of catechin equivalents per gram of sample (mg of CE/g).

Characterization of Phenolic Compounds. Analyses of phenolic compounds were performed on a Finnigan Surveyor Modular HPLC system (Thermo Electron Co.). Chromatographic separation of the compounds was achieved using an analytical column, Luna 3 μm C18 (2), 150×2.0 mm, and a guard column, Security Guard C18 (ODS), 4×3.0 mm i.d. (Phenomenex, Inc., Torrance, CA) at a flow rate of 0.2 mL/min. Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: from 0 to 15 min by 5% B, from 15 to 50 min by 5−40% B, and from 50 to 55 min by 40−95% B with a linear gradient, followed by 55−65 min by 95% B isocratic. The photodiode array detector (PDA) was operated at wavelengths between 220 and 400 nm. The system was coupled to a Finnigan LCQ Advantage MAX ion trap mass spectrometer and operated in electrospray ionization (ESI) mode. Samples of 20 μL of extracts were directly injected into the column using a Rheodyne (model 7725i) injection valve. ESI source and negative ionization mode were used with different fragment voltages. Nitrogen was used as the neutralizing and drying gas. The typical operating parameters were as follows: spray needle voltage, 5 kV; ion transfer capillary temperature, 300 °C; nitrogen sheath gas, 40; and auxiliary gas, 5 (arbitrary units). The ion trap contained helium damping gas that was introduced in accordance with the manufacturer's recommendations. Mass spectra were acquired in a *m/z* range of 100−1000, with 5 microscans and a maximum ion injection time of 200 ms. The SIM analysis was a narrow scan event that monitored the *m/z* values of the selected ion in a range of 1.0 Th centered on the peak for the molecular ion; this function was used in the analysis of molecular ions of the phenolic compounds in extracts using negative ESI modes (*15*, *16*).

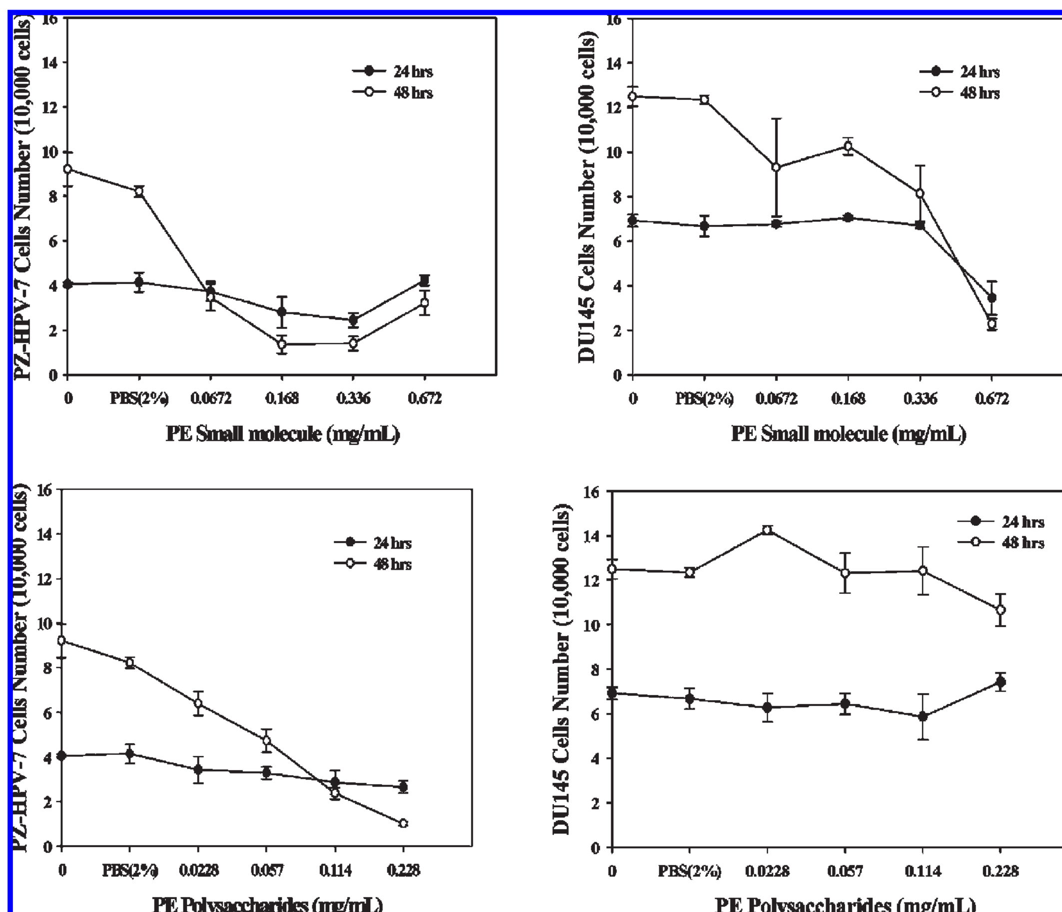

Figure 3. Effect of soluble plysaccharide and small molecular polyphenolics of PE on cell viability of both PZ-HPV-7 and DU-145 cells: effect of small molecular phenolics fraction on PZ-HPV-7 cells (upper left) and DU-145 cells (upper right); effect of soluble polysaccharides on PZ-HPV-7 (lower left) and DU-145 cells (lower right). The dosages used were equivalent to the original soluble polysaccharide or small molecular polyphenolic contents in 0.1, 0.25, 0.50, and 1.0 mg/mL, respectively (refer to **Figure 1**). PBS, phosphate buffer solution.

Cell Viability. An MTT assay was performed mainly by following the method of Chen et al. (*10*) with slight modification by the manufacturer (Bio-Tek Instruments, Winooski, VT). In brief, cells were seeded onto a 24-well plate at 2×10^4 cells/well. Required amounts of the soluble fraction of polysaccharides (P_{ws}) and the small molecular fraction (Φ_{sm}) (**Figure 1**) of PE were respectively dissolved in PBS and heated at 50 °C for 30 min. After cooling, the PBS solutions were added to respective cell culture mediums (for DU-145 cells, DMEM, 10% FBS, 1% PS, 1% NEAA; for PZ-HPV-7 cells, SFM medium, 1% PS) to obtain concentrations at 0, PBS 2%, 0.0228, 0.057, 0.114, 0.228 mg/mL, respectively, for P_{ws}, and 0, PBS 2%, 0.0672, 0.168, 0.336, and 0.672 mg/mL, respectively for Φ_{sm}. The media were inoculated respectively with DU-145 and PZ-HPV-7 cells and incubated at 37 °C under a 5% CO_2 atmosphere for 48 h. During the cultivation period, MTT assays were performed at 24 and 48 h, respectively. The optical density was measured at 570 nm.

RESULTS AND DISCUSSION

Yield of Polysaccharides. The water-soluble polysaccharide content (P_{ws}) in PE was 22.8%, whereas the soluble polyphenolics (Φ_{sm}) had a fraction of 67.2% (**Figure 1**), implicating a relatively huge mount of soluble polysaccharide in PE, which possibly could be associated with the anticancer bioactivity as often cited elsewhere for many herbal polysacchrides (*14*).

Molecular Mass Determination. As is well-known, the optical density measured at 490 nm resulting from phenol−sulfuric acid reaction accounts for polysaccharide and that at 280 nm corresponds to protein content. The carbohydrate and protein contents in P_{ws} were 29.06 and 10.27% w/w, respectively, to yield a ratio of 2.83 (w/w), characteristically indicating a peptidoglycan in nature. The averaged molecular mass was 5029 kDa (**Figure 2**).

Composition of Water-Soluble Polysaccharides. PE uniquely possesses a novel rhamnoallan peptidoglycan that has never been cited. This novel rhamnoallan was a hemicellulose structurally consisting of tremendous amounts of rhamnose and allose, together with moderate amounts of arabinose and tallose (**Table 1**). The water-soluble polysaccharide fraction of PE was composed of nine different monosacchrides, predominantly rhamnose (36.05 mol %) and allose (24.24 mol %) in a molar ratio of 3:2. Such a huge amount of allose was for the first time found in herbal preparations. Hence, this peptidoglycan was uniquely named herein "rhamnoallosan". To our knowledge, we are the first to report the existence of this novel peptidoglycan (**Table 2**). In addition, the peptide fraction contained 14 amino acids, among which, the main amino acids were glycine, leucine, proline, and alanine. Their yields were 37.12, 12.68, 10.05, and 8.97 wt %, respectively (**Table 1**). A comparison of the monosaccharide composition in other polysaccharides and that of PE is listed in **Table 2**. Literature elsewhere documented many categories of polysaccharides present in herbal preparations, most of which contained D-glucose (*13*, *17*), L-arabinose, D-galactose (*17*, *18*), and D-mannose (*13*, *16*), existing in a diversity of structures including rhamnogalacturonan (*18*), arabinogalactan (*19*), and arabinoxylan (U.S. Patent 5560914, Daiwa Pharm., Co., Ltd., Tokyo, Japan) etc.

Contents of Polyphenolics and Flavonoids. The total polyphenolic and flavonoid contents in guava leaves previously reported

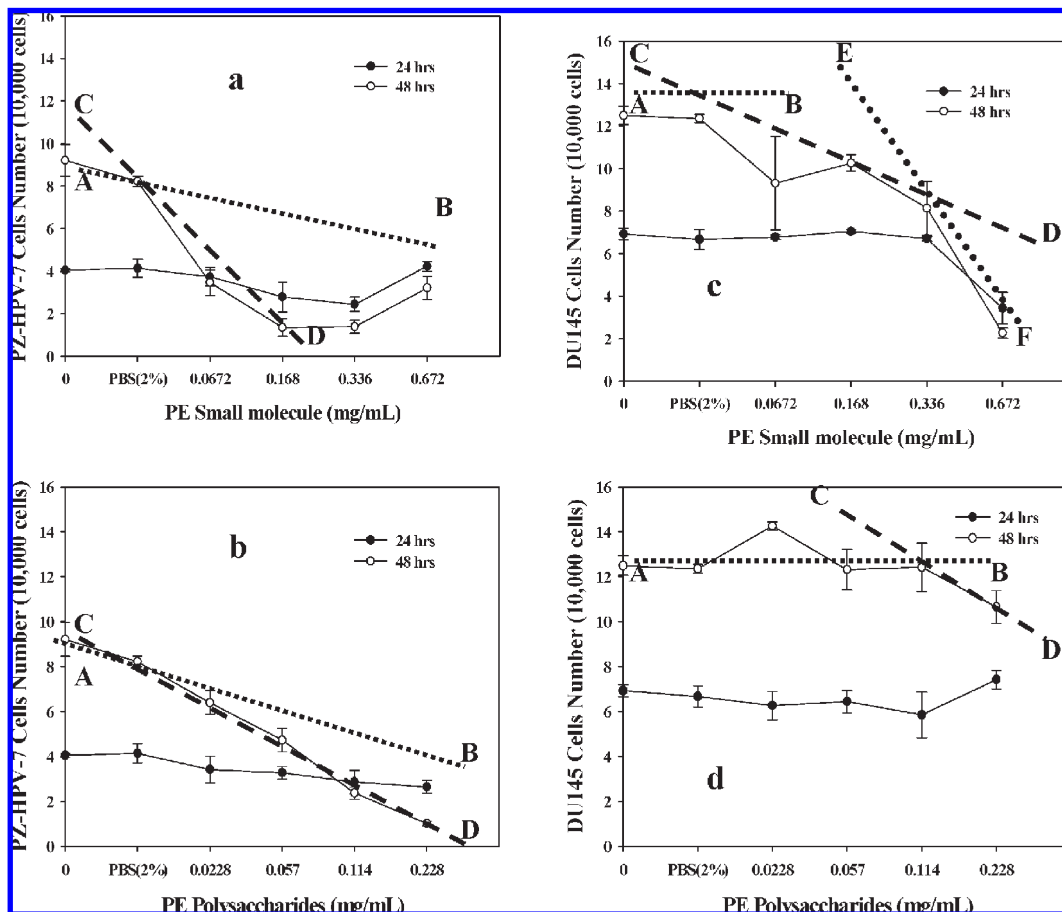

Figure 4. Death rates of both PZ-HPV-7 and DU-145 cells affected by soluble polysaccharide and small molecules of PE: effect of small molecular phenolics fraction on PZ-HPV-7 cells (**a**) and DU-145 cells (**c**); effect of soluble polysaccharides on PZ-HPV-7 (**b**) and DU-145 cells (**d**). The dosages used were equivalent to the original soluble polysaccharide or small molecular polyphenolic contents in 0.1, 0.25, 0.50, and 1.0 mg/mL, respectively (refer to **Figure 1**). PBS, phosphate buffer solution.

Table 5. Different Death Rate Kinetic Parameters for PZ-HPV-7 and DU-145 Cells Affected by either P_{ws} or Φ_{sm}[a]

	kinetic parameters			
cell line/medicine	k_{pbs}[b] (h^{-1})	k_c[c] (cells mg^{-1})	k_t[d] (h^{-1})	k_{app}[e] (cells mg^{-1} h^{-1})
PBS	1.67×10^2	1.43×10^5	5.00×10^2	2.98×10^3
PZ-HPV-7 Φ_{sm}	1.67×10^2	3.04×10^5	1.06×10^3	6.32×10^3 (7.75×10^2)[f]
PZ-HPV-7 P_{ws}	1.67×10^2	2.11×10^5	5.00×10^2	4.39×10^3 (2.28×10^3)[f]
DU-145 Φ_{sm}	0.0	1.93×10^5 (phase 2 along curve CD)	6.77×10^2 (phase 2 along curve CD)	4.03×10^3 (phase 2 along curve CD)
		1.70×10^5(phase 3 along curve EF)	1.19×10^3 (phase 3 along curve EF)	3.53×10^3 (phase 3 along curve EF); (3.26×10^3)[f]
DU-145 P_{ws}	0.0	1.40×10	3.33×10^2	2.92×10^3 (9.60×10^3)[f]

[a] P_{ws}, water-soluble polysaccharides in PE; Φ_{sm}, small molecular phenolics in PE. Data were calculated from **Figure 4**. [b] k_{pbs} calculated from eq 1. [c] k_c from eq 3. [d] k_t from eq 5. [e] k_{app} from eq 6. [f] Data depicted from Chen et al. (*10*).

were 156.6 mg of GAE/g and 82.9 mg of CE/g (*6*), respectively. In the lyophilized aqueous extract of *P. guajava* budding leaves (PE), the contents were effectively conserved, yielding 470.0 mg of GAE/g of PE and 248.6 mg of CE/g of PE, respectively, for polyphenolic and flavonoid contents (**Table 3**), being comparable to those of Edmundo et al. (*20*), who indicated that guava fruits showed, per gram, a ferric reducing/antioxidant power (FRAP) of 1300 mM and a polyphenolic content of 330 mg of GAE. PE contained gallic acid, catechin, epicatechin, rutin, and quercetin to reach 348, 102, 60, 100, and 102 mg/g, respectively. In addition, minute amounts of naringenin and kaempherol were also present (**Table 3**). Such a high content of polyphenolics was unsurpassed ever by any other herbal preparation. Quercetin and its monophenolic metabolites can inhibit autoxidation, glycation, and glycoxidation of carbohydrates as evidenced by a synergism when quercetin was used in combination with its metabolites. As demonstrated elsewhere, quercetin, rutin, and protocatechuic acid are the common strong major antioxidants in many herbs (*21*) (also refer to **Table 4**). In vitro data suggested that a flavonoid concentration of 1 μmol per liter of blood is sufficient to be therapeutically effective in vivo (*22*, *23*). In addition, Nakagawa et al. (*5*) reported that the gallate moiety in tannin can efficiently suppress protein oxidation and glycation in vitro.

In Vitro Anticancer Bioactivity. With PE at 1 mg/mL, correspondingly containing water-soluble polysaccharides P_{ws} 0.228 mg/mL and water-soluble polyphenolics Φ_{sm} 0.672 mg/mL, the DU-145 cell viability was suppressed to 84.1 and 15.9%, respectively (right column in **Figure 3**). In contrast, PZ-HPV-7 cells followed an autodecaying process in PBS after incubating for

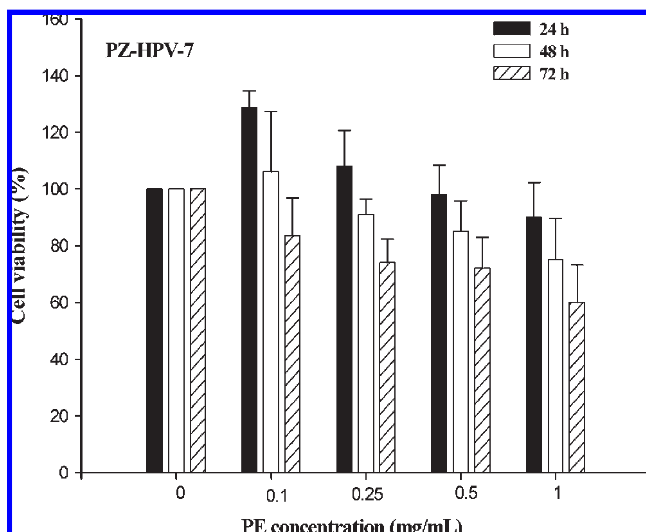

Figure A2. Stable survival rates of PZ-HPV-7 cells as previously reported. As can be found, >85% survival was seen at 0.5 mg of PE/mL after incubation for 48 h. Reprinted from ref (*10*). Copyright 2007 Lawrence Erlbaum Associated.

48 h (**Figure 3**, left column), which was not found in DU-145 cells (**Figure 3**, right column). Hence, the death rate of PZ-HPV-7 cells in the presence of P_{ws} and Φ_{sm} was corrected for such an effect (**Figure 4a**,**b**). Astonishingly, the small molecular polyphenolics of PE affected DU-145 cells differently in three responsive phases, that is, phase 1 (curve AB), phase 2 (curve CD), and phase 3 (curve EF) (**Figure 4c**), which was totally unseen in PZ-HPV-7 under the same testing conditions (**Figure 4a**,**b**) and in DU-145 cells in the presence of P_{ws} (**Figure 4d**).

The corresponding kinetic coefficients were analyzed from **Figure 4** and are given in **Table 5**.

Assuming the death rate of cells obeys first-order kinetics, the blank death rate kinetics in the absence of either P_{ws} or Φ_{sm} would be the slopes along curves AB; thus, we have

$$-(\mathrm{d}N/\mathrm{d}t)_{\mathrm{pbs}} = k_{\mathrm{pbs}}N \qquad (1)$$

where $-(\mathrm{d}N/\mathrm{d}t)_{\mathrm{pbs}}$ denotes the decay rate in phosphate buffer solution, whereas the concentration−responsive death rate at a defined time interval in the presence of either P_{ws} or Φ_{sm} would be the slopes along curves CD, that is

$$-(\mathrm{d}N/\mathrm{d}C)_t = k_c N \qquad (2)$$

On rearrangement, eq 2 gives

$$-(\mathrm{d}N/N)_t = k_c\,\mathrm{d}C \qquad (3)$$

Similarly for the time-responsive death kinetics, we have

$$-(\mathrm{d}N/\mathrm{d}t)_c = k_t N \qquad (4)$$

Rearrangement of eq 4 leads to

$$-(\mathrm{d}N/N)_c = k_t\,\mathrm{d}t \qquad (5)$$

Combination of eqs 3 and 5 yields the apparent overall kinetics

$$-\int_{N_0}^{N}(\mathrm{d}N/N)_{\mathrm{app}} = k_{\mathrm{app}}\int_0^c \mathrm{d}C\int_0^t \mathrm{d}t \qquad (6)$$

Thus, we arrived at the concentration- and time-responsive apparent overall kinetics that can be used to evaluate the death rate of cancer cells (from a population of N_0 decreased to N) when treated with the aqueous extract of *P. guajava* budding leaves (PE) at a concentration of dC for a very short time interval of dt, provided the kinetic parameter k_{app} is known. The corresponding kinetic coefficients were analyzed from **Figure 4** and are given in **Table 5**. **Table 5** reveals that the death kinetic rates of both cell lines seemed to be very comparable. Due to the instability of PZ-HPV-7 cells in PBS, cell mortality was significantly affected, as evidenced by the kinetic analysis for PBS alone (**Table 5**). Moreover, speculatively such an instability could get worse for a longer incubation (**Figure 3**; see the slope along curve AB in **Figure 4a**,**b**), which was totally not found in our previous study ((*10*); also refer to Appendix **Figures A2** and **A3**) or in present DU-145 cells. In view of therapeutic logics, the cytotoxicity of any anticancer medicines should be significantly more harmful to the cancer cells than to the normal cells. Hence, the kinetic data for PZ-HPV-7 cells obtained from this paper were not reliable in this regard (**Table 4**). Alternatively, the reconstructed data from previously reported ((*10*); Appendix **Figure A2**) were adopted. When treated with Φ_{sm} at dosages below or equal to 0.5 mg of PE/mL, the feasible values of k_{app} would be 7.75×10^2 and 4.03×10^3 cells mg^{-1} h^{-1}; conversely when applied with P_{ws}, the acceptable values of k_{app} became 2.28×10^3 and 9.60×10^3 cells mg^{-1} h^{-1} for PZ-HPV-7 cells and DU-145 cells, respectively ((*10*); Appendix **Figure A2**, data denoted by footnote *f* in **Table 5**). Apparently, our previous data showed that PE was almost 3-fold more harmful to the DU-145 cells than to the PZ-HPV-7 cells with respect to the cytotoxicity of soluble polysaccharide P_{ws}. However, the small molecular weight fraction Φ_{sm}, which stands for the polyphenolic and flavonoid fraction, was seen to possess comparable bioactivity in this regard.

To apply these kinetic findings, we propose a predictive equation for the estimation of cancer cell death rates.

$$-(\mathrm{d}N/N)_{\mathrm{est}} = -[k_{\mathrm{app},\Phi\mathrm{sm}}W_{\Phi\mathrm{sm}} + k_{\mathrm{app,Pws}}W_{\mathrm{Pws}}] \qquad (7)$$

where the term $-(\mathrm{d}N/N)_{\mathrm{est}}$ is the estimated cancer cell death rate with respect to the initial cell population N_0, $k_{\mathrm{app},\Phi\mathrm{sm}}$ is the apparent cell death kinetic coefficient of the soluble small molecules, and $k_{\mathrm{app,Pws}}$ is the apparent cell death kinetic coefficient of the water-soluble polysaccharides. $W_{\Phi\mathrm{sm}}$ and W_{Pws} are the weights of soluble small molecules and soluble polysaccharides expressed in milligrams in the medicated dosage, respectively.

Substitution of the kinetic parameters k_{app} of DU-145 in **Table 5** into eq 7 leads to

$$-(\mathrm{d}N/N)_{\mathrm{est}} = -[(4.03\times10^3)W_{\Phi\mathrm{sm}} + (9.60\times10^3)W_{\mathrm{Pws}}] \qquad (8)$$

for dosages of PE of ≤0.5 mg/mL and to

$$-(\mathrm{d}N/N)_{\mathrm{est}} = -[(3.53\times103)W_{\Phi\mathrm{sm}} + (9.60\times10^3)W_{\mathrm{Pws}}] \qquad (9)$$

for dosages of PE of >0.5 mg/mL.

Suggestively, the better dosages to be taken were at concentrations ≤0.5 mg of PE/mL (**Figure 4**). Correspondingly, the dosages of P_{ws} and Φ_{sm} in PE to be taken are 0.114 and 0.336 mg/mL, respectively (**Figures 3** and **4**). A safety test conducted previously indicated that 2.0 g of PE/kg of body weight was totally nontoxic to Sprague−Dawley rats (*1*). For a person having a blood volume of 4000 mL to fully elevate the plasma effective concentration of PE to reach a plasma level of 0.5 mg/mL, a single oral dosage of 2000 mg is apparently required, which obviously is still far below the toxic level.

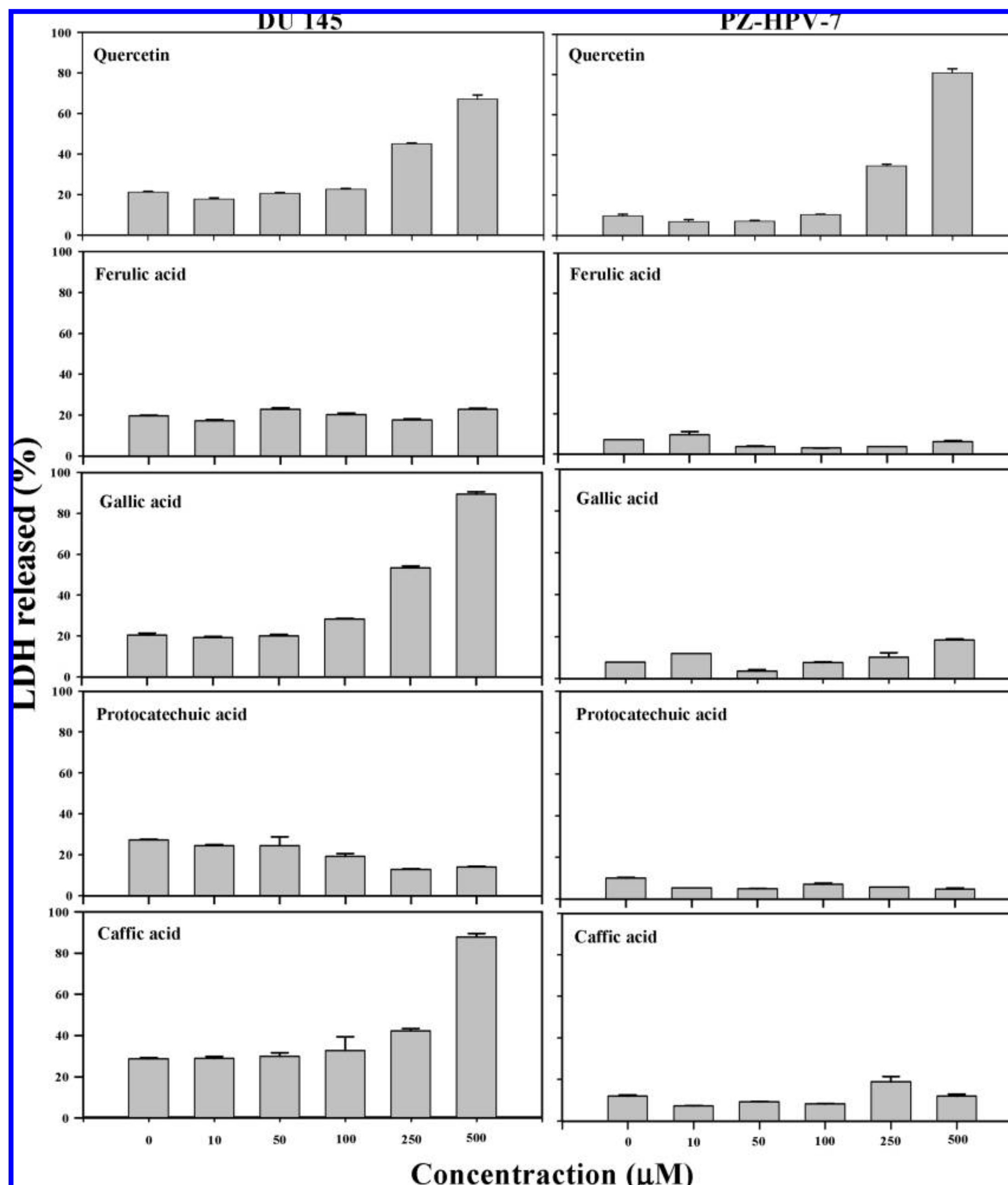

Figure A3. Influence of phenolic compounds on released LDH in DU-145 and PZ-HPV-7 cells. DU 145 and PZ-HPV-7 cells were incubated with phenolic compounds at 37 °C for 2 days. Courtesy of Professor C.-L. Hsieh, 2008, unpublished data.

Conclusively, in PE we found a novel peptidoglycan rhamnoallosan, which has revealed strong anti-DU-145 bioactivity complementary to coexisting soluble polyphenolics.

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