We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



8

Fractionation and Characterization of Bioactive Components in Kefir Mother Culture that Inhibit Proliferation of Cultured MCF-7 Human Breast-Cancer Cells

Chujian Chen[1,2], Hing Man Chan[3] and Stan Kubow[1]
[1]*School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Quebec*
[2]*School of Public Health, Nantong University, Nantong, Jiangsu*
[3]*Community Health Program, University of Northern British Columbia, Prince George, British Columbia*
[1,3]*Canada*
[2]*People's Republic of China*

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women. Resistance to therapy is the major reason for failure of cancer treatment (Mesner *et al.*, 1997). There is a critical need to identify new chemotherapeutic agents that can increase susceptibility to anti-breast cancer drugs or overcome resistance mechanisms, which would improve patient outcomes, prevent relapse, and prolong patient survival. Kefir is an acidic and mildly alcoholic fermented milk that originated in the Caucasian mountains of former Soviet Union, and enjoys a rich tradition of health benefits. Consumption of fermented dairy foods has been associated with lower incidence of breast cancer (Ronco *et al.*, 2002). Research on the putative health benefits of fermented milks suggested that by-products of bacterial fermentation of proteins, lipids and carbohydrates present in fermented milks exert health benefits beyond basic nutrition including anti-tumor action, immune system enhancement and antioxidant effects (Parvez *et al.*, 2006). Conjugated linoleic acid (Schonberg *et al.*, 1995), sphingolipids (Dillehay *et al.*, 1994), polysaccharides (Shiomi *et al.*, 1982), organic acids (Garrote *et al.*, 2000) and some proteins and peptides (Svensson *et al.*, 1999) have been shown to have antimutagenic and antitumor effects. Promising results regarding anti-tumor activity of yogurt extracts in cell culture (Biffi *et al.*, 1997) and kefir extracts in animal feeding studies (Cervikbas *et al.*, 1994; Furukawa *et al.*, 2000; Shiomi *et al.*, 1982) have been reported. However, the bioactive components in kefir and the mechanisms involved in the various biofunctional effects of kefir are still not well characterized, particularly in terms of anti-cancer activities. In our recent studies, kefir extracts exerted potent anti-proliferative effects on cultured human mammary tumor cells as compared to extracts of yogurt or milk (Chen *et al.*, 2007). The aim of the present study was to fractionate and characterize bioactive components in kefir mother culture that exert antiproliferative effects in MCF-7 cells.

2. Materials and methods

Kefir samples were provided by Liberte Inc. (Brossard, Canada). The large-scale production of kefir involves a two-step fermentation process. The first fermentation is achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting around 24 h, the grains are removed by filtration. The filtrate (kefir mother culture) is added to milk (1-3%), which is further fermented for 24 h and packaged for the consumer market (final kefir commercial product). Samples from three different batches were used. Upon receipt of the samples, they were immediately well stirred, and centrifuged at 4°C, 32,000 x g, for 60 min (Sorvall RC 5C Centrifuge, rotor ss-34, Sorvall Instruments, Wilmington, USA). The supernatant was filtered with a 0.45 m membrane filter followed by a 0.2 m filter. The filtrates were stored at −80°C for future use.

2.1 Macronutrients and minerals

A Flexi-Dry MP lyophilizer (FTS Systems Inc., Stone Ridge, USA) was used for triplicate determination of moisture. Ten grams of homogenized sample was transferred into pre-weighed aluminium weigh boat, frozen at -80°C for approximately 1 h and then freeze dried for 48 h. The boat was weighed again and the moisture was calculated. A LECO FP-428 Nitrogen Determination System (LECO Corporation, St. Joseph, USA) was used to determine nitrogen content in triplicate for freeze-dried samples. Crude protein content was calculated using a conversion factor of 6.25. Protein in solution was determined by using Bio-Rad protein assay kit according to the instruction with the kit (Bio-Rad Laboratories, Hercules, USA). Peptide concentrations were analyzed by the method of Church *et al.* (1983) (opthialdehyde; OPA). Crude fat was analyzed in triplicate with an automatic Soxtec extraction system (Soxtec HT6 Tecator AB, Hoganas, Sweden). Three grams of freeze-dried, well-mixed sample was loaded and analyzed. Proper amount of freeze-dried samples were digested in 70% (w/v) nitric acid (Fisher Scientific, trace metal grade) and minerals (i.e., Ca, Mg, Zn, Fe, Na and K) were determined by using Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer (Nissei Sango Ltd., Mississauga, ON, Canada).

2.2 Organic acids

Lactic acid content was analyzed using a Sigma lactate kit assay (Sigma Diagnostics, Cat. No. 735-10, St. Louis, USA). Organic acids were determined by HPLC according to the method of Guzel-Seydim *et al.* (2000). Five mL of each sample was diluted with 25 mL 0.01 M H_2SO_4, vortexed for 1 min followed by centrifugation at 2000 x g for 10 min. Supernatants were collected and filtered through 0.2 m filter. Volumes of 20 l of samples and standards were injected into a Beckman Gold HPLC system (Beckman Coulter, Fullerton, USA) equipped with an Aminex HPX-87H (300 mm x 7.8 mm) organic acid column (Bio-Rad Laboratories, Hercules, USA). Degassed 8 mM sulfuric acid (H_2SO_4) was used as the mobile phase. The organic acids oxalate, citrate, malate, succinate, formate and acetate were detected at 215 nm. Organic acids were quantified using external standards (organic acid analysis standard, Bio-Rad Laboratories, Hercules, USA).

2.3 Molecular weight cut-off fractionation (MWCO)

Centriplus centrifugal filter devices were used to get MWCO fractions at 3000 Da (Millipore, Bedford, USA). Ten milliliters whole extract were loaded to the sample reservoir and the

assembled device was centrifuged at 4°C, 3000 x g for 290 min. The filtrates were collected for further analysis.

2.4 Size exclusion HPLC (SEC) separation

One hundred microliters of kefir mother culture extract was injected into a TSK G2000SWXL column (78 mm x 30 mm, SUPELCO, Bellefonte, USA) and separated with a Shimadzu LC-6AD Liquid Chromatograph system (Shimadzu Scientific Instruments, Inc. Columbia, USA) with UV detection at 210 nm. The separation buffer used was a mixture of 45% acetonitrile in 0.1% trifluoroacetic acid (TFA) with a flow rate of 0.4 ml/min for 40 min. Nine fractions were collected for each HPLC run, and fractions from five to ten runs were pooled. The above nine fractions were evaporated with N_2 and then freeze dried, stored at −80°C for cell culture incubations and for further analytical analyses.

2.5 Reverse phase HPLC (RP-HPLC) fractionation

The fraction(s) collected with SEC HPLC that showed antiproliferative effects on MCF-7 cells were further analyzed with a Prosphere 300 C4 column (5 m, 250 mm x 4.6 mm) (Alltech Associate, Inc. Deerfield, USA) using a Beckman Gold HPLC System (Beckman Coulter, Fullerton, USA). After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 ml per min, the fractions were eluted with a linear gradient of buffer A (0.1% TFA in water) and buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Dual channel absorbance was monitored at 210 nm (channel A) and 280 nm (channel B).

2.6 Preparative HPLC fractionation

Three batches of extracts of kefir mother culture were pooled and fractionated using the Centriplus centrifugal filter devices to obtain fractions of compounds with molecular weights less than 3000 Da. The fractions of MWCO less than 3000 Da were freeze-dried using a FLEXI-DRY MP Freeze Dryer (FTS Systems, Inc. Stone Ridge, U SA). Five g of the lyophilized MWCO fractions were dissolved in 20 ml of water. Ten milliliters of reconstituted solution were loaded on a C4 preparative column (300 Å, 5 m, 300 mm x 50 mm) (Vydac Company, Herperia, CA) and separated with a Water Delta Prep 4000 HPLC system (Waters Corporation, Milford, USA). After the column was equilibrated with buffer A (0.6% acetic acid in water) at a flow rate of 13 ml and the fractions were eluted with a linear gradient of buffer A and buffer B (0.6% acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B. A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals. The fractions were then lyophilized and kept at −80°C for further cell culture and analysis. The fractions were reconstituted by adding water and peptide and protein concentrations were determined before cell culture assays.

2.7 Capillary electrophoresis

Capillary zone electrophoresis (CE) was performed using a P/ACE ™ 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to an IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated fused silica capillary column (57 cm × 50 m, the length from intake to detector was 50 cm) was assembled in the P/ACE cartridge (Polymicro

Technologies, Phoenix, Arizona USA) for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time. The capillary was flushed with 1 M sodium hydroxide, followed by nanopure water, 0.1 M sodium hydroxide, nanopure water, 1 M hydrochloric acid, and again water, each for 5 min at a pressure of 40 psi. A solution of 5% polybrene and 2% ethylene glycol was then passed for 10 min at 40 psi. Excessive coating was then removed by flushing with water for 2 min. Further capillary flushing was then performed for additional 10 min using 200 mM formic acid buffer. Before each sample application, the capillary was rinsed with 1 min water, 1 min 0.1 M sodium hydroxide, 1 min water and 3 min separation buffer (200 mM formic acid in water, pH 2.0). After the completion of each run, the capillary was rinsed with nanopure water for 1 min, 0.1 M sodium hydroxide for 1 min and nanopure water for 1 min. Peptide standard and sample injections were carried out at the anode end of the capillary using N_2 pressure (0.5 psi) for 5 sec and was separated at a constant temperature of 20°C with a 200 mM formic acid (pH 2.0) as separation buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation. The capillary was re-coated after every 5 runs.

2.8 MALDI-TOF for estimation of molecular weight

Reconstituted SEC-HPLC and RP-HPLC fractions were analyzed using a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Palo-Alto, CA, USA) with a laser at 337 nm and an acceleration voltage of 20.000 V.

2.9 Mass spectrometry

Mass spectrometric analysis in the positive ion mode was performed on a triple quadupole mass spectrometer (SCIEX API III Biomolecular mass analyzer, Thornhill, Ontario, Canada). Lyophilized reversed phase HPLC fractions having antiproliferative effect on MCF-7 cells were reconstituted in 0.5 mM ammonium acetate in methanol or in 10% acetic acid in 20% aqueous methanol. The resulting solution was then infused into the electrospray ion-source by a syringe pump (Harvard Apparatus Model 22, South Natick, MA) at a flow rate of 1.5 ml/min. The ion-spray voltage was set at 5.5 kV and the orifice potential was set at 50 V. Argon was used as the collision gas at a collision gas thickness (CGT) of 1.5×10^{14} for collision-induced fragmentation MS-MS analysis.

2.10 Cell Culture screening for antiproliferative effects

MCF-7 cells were purchased from ATCC (ATCC, Manassas, USA). Cells were routinely propagated as a monolayer culture in Dulbcco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), in a 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3-4 days. Normal human mammary epithelial cell lines (HMEC) were graciously provided by Dr. M.R. Stampfer (Lawrence Berkeley National Laboratory, Berkeley, USA). Cells were routinely propagated as a monolayer culture in Mammary Epithelial Growth Media (MEGM, Clonetics, San Diego, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passaged every week. For the experiments, both

MCF-7 and HMEC cells were harvest from the dish using 0.25% trypsin-EDTA solution (Sigma, St Louis, MO, USA).

2.11 Cell proliferation experiments in 24-well plates

Cells previously harvested were seeded in 24-well plates, i.e., 10,000 cells for MCF-7 per well in DMEM supplemented with 10% FBS and 5,000 cells for HMEC per well in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. To study the dose response, a serial dilution of each the extract using the culture media was made to achieve final concentrations of extracts at 5%, 2.5%, 1.3%, 0.6%, and 0.3% (vol./vol.), respectively. Because the kefir extracts were acidic (approximately pH 4.5), Dulbecco's Phosphate Buffered Saline (PBS) buffer (Gibco BRL, Grand Island, NY) was added to the culture media to adjust the pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d. Cell nuclei were counted in order to eliminate the difficulty in counting whole cells due to clumping. Furthermore, a more uniform distribution of nuclei over the grids of a hemacytometer was seen compared with whole cells. Media was aspirated from wells and cells were rinsed with 500 l of PBS. 250 l of hypotonic buffer (0.01 M HEPES, 1.5 M $MgCl_2$, pH 7.5) was added to each well. After 2 min, 250 l of cell lysing solution (10% ethyl hexadecyl dimethyl ammonium bromide, 3% glacial acetic acid, in water) was added. The plate was shaken lightly every minute for 5 min. Cell lysis was confirmed microscopically as indicated by a suspension of clean nuclei. The suspension was mixed and the nuclei were counted using a Coulter Counter (Coulter Counter Corporation, Fullerton USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatment was expressed as a percent of control.

2.12 Cell proliferation experiments in 96-well plates

Cells previously harvested were seeded in 96-well plates at 1,000 cells per well for MCF-7 in DMEM supplemented with 10% FBS and for HMEC in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. A serial dilution of each test fraction was made to study the dose-response. PBS buffer was added to the culture media to keep the final pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d and the cell numbers in each well were determined by using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatments was expressed as a percent of control.

2.13 Statistics

All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC USA). Means were compared with Student's t test. Two-way ANOVA was used to analyze the effects of treatments and doses for the cell culture experiments. The differences among doses and treatments were determined by the Student-Newman-Keuls (SNK) multiple comparison test. Statistical significance was considered at $P < 0.05$.

3. Results

Moisture, macronutrients and some minerals in kefir mother culture and final kefir described in Table 1.

Component	Batch 1		Batch 2		Batch 3	
	Mother culture	Kefir	Mother culture	Kefir	Mother culture	Kefir
Moisture (g)	[1]89.78±0.09	90.40±0.06	91.22±0.19	90.03±0.02	89.56±0.07	90.17±0.08
Ash (g)	0.68±0.005	0.67±0.004	0.66±0.006	0.70±0.003	0.68±0.002	0.67±0.003
Protein (g)	3.05±0.16	2.81±0.03	2.78±0.03	3.14±0.04	3.28±0.01	2.77±0.02
Fat (g)	2.32±0.06	1.77±0.04	2.46±0.06	1.46±0.02	2.36±0.04	1.48±0.01
Calcium (mg)	78.20±0.66	77.70±0.71	78.93±0.58	77.40±0.64	81.53±0.73	80.40±0.56
Iron (mg)	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001
Zinc (mg)	0.28±0.02	0.29±0.02	0.28±0.02	0.30±0.02	0.31±0.03	0.29±0.03
Magnesium (mg)	8.42±0.55	8.65±0.35	8.88±0.56	8.40±0.71	8.97±0.48	8.70±0.42
Sodium (mg)	28.51±1.86	29.0±0.71	28.60±2.03	30.05±2.05	30.99±1.08	31.05±0.72
Potassium (mg)	60.28±0.89	60.60±0.42	59.36±1.16	61.30±1.34	60.40±1.08	60.95±0.78

Table 1. Nutrient composition from three different batches of kefir mother culture and final commercial kefir product (per 100 g wet weight) ([1]Mean±SD, n=3).

No significant differences among nutrients and organic acids were found, except that lactic acid in the kefir commercial product was higher than that of kefir mother culture (Table 2).

Component	Mother culture	Kefir
Lactic acid[1]	[3]77.86±8.94	106.87±9.36*
Uric acid [2]	0.014±0.003	0.012±0.002
Pyruvic acid[2]	0.54±0.15	0.52±0.10
Oxalic acid[2]	[4]ND	ND
Citric acid[2]	3.27±0.17	3.22±0.28
Malic acid[2]	54.79±9.25	57.62±11.01
Succinic acid [2]	1.61±0.24	1.71±0.08
Formic acid[2]	Not detectable	Not detectable
Acetic acid[2]	3.81±0.74	3.46±0.47

Table 2. Organic acid concentrations (mmol/l) in mother culture and final kefir commercial product ([1]Determined by lactic acid kit assay method; [2]Determined by HPLC method of Guzel-Seydim *et al.* (2000); [3]Mean±SD, n=3; [4]ND = Not detectable; *$P < 0.05$).

Capillary electrophoresis analyses showed that the profiles of extracts of mother culture and kefir were similar (data not shown). In our earlier study, the extract of kefir mother culture showed a tendency of having stronger antiproliferative effect on MCF-7 cells than other extracts (Chen *et al.*, 2007). Hence, mother culture was chosen for further fractionation and cell culture tests to identify the bioactive component(s). Extracts of kefir mother culture that were fractionated via the 3000 Da MWCO were analyzed for protein and peptide content (Table 3).

Samples	Moisture (g/100g)	Crude protein[1] (g/100g)	Protein[2] (g/ml)	Peptides[3] (mmol/l)
Whole extract	[4]96.39±0.24	0.27±0.03	152.91±11.53	5.53±0.26
MWCO<3000	[5]ND	ND	5.10±0.07*	4.21±0.32*
MWCO>3000	ND	ND	195.94±4.11*	7.16±0.36*

Table 3. Protein and peptide concentrations in different MWCO fractions of extracts of kefir mother culture ([1]Determined by using Nitrogen Determinator; [2]Determined by Bradford method (1976); [3]Determined by OPA method of Church *et al.* (1983); [4]Mean±SD, n=3; [5]ND: No data is available; *Significant different at $P < 0.05$ when compared to protein or peptide concentration in whole extract).

Both protein and peptide concentrations were significantly lower in the fraction of MWCO less than 3000 Da ($P < 0.05$) relative to the whole kefir mother culture extract. Both the whole extract and the fraction with the MWCO less than 3000 Da were screened by MCF-7 cell culture using 24-well plates. As shown in Figure 1, the filtrate of MWCO less than 3000 Da had a dose dependent antiproliferative effect on MCF-7 cells comparable to that of the whole mother culture extract.

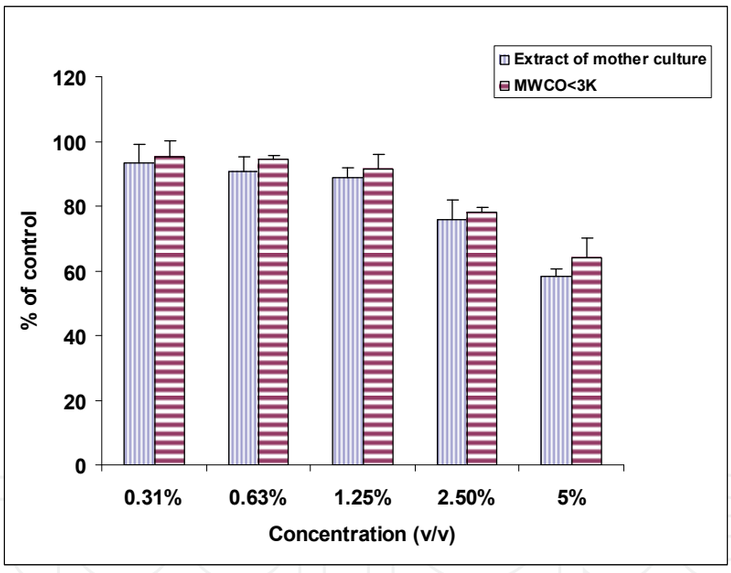


Fig. 1. Antiproliferative effects on MCF-7 cells induced by extracts of: (A) whole kefir mother culture; and (B) fraction of kefir mother culture obtained with MWCO less than 3000 Da. Cell proliferation was analyzed in 24-well plate. Values were graphed as Mean±SD, n=4.

The kefir mother culture filtrate that contained the MWCO less than 3000 Da was further separated with SEC-HPLC. Nine fractions were collected with each run with retention times of 14.6 to 17.0, 17.1 to 19.0, 19.1 to 21.0, 21.1 to 23.7, 23.8 to 25.6, 25.7 to 27.3, 27.4 to 29.1, 29.2 to 31.6, 31.7 to 33.7 min, respectively. Ten runs of SEC-HPLC fractions were pooled, freeze-dried and reconstituted in 2 ml water. The estimated molecular weight, protein and peptide concentrations are shown in Table 4. All the nine fractions were tested with MCF-7 cells in 96-well plates for their antiproliferative effects.

Fraction	Retention time (min)	Molecular Weight (Dalton)	Protein (g/ml)	Peptides (M/l)
1	14.4-17.0	13116-29507	145.7±5.12	274.53±2.19
2	17.1-19	7618-12745	118.56±4.89	284.70±5.31
3	19.1-21	4672-7425	43.62±2.84	208.33±2.26
4	21.1-23.7	2588-4565	16.80±1.56	491.14±6.67
5	23.8-25.6	1776-2535	3.23±0.26	422.01±5.17
6	25.7-27.3	1297-1742	2.92±0.2	655.11±4.58
7	27.4-29.1	949-1274	1.38±0.12	1168.33±8.22
8	29.2-31.6	635-934	4.77±0.16	1053.71±5.17
9	31.7-33.7	470-625	3.84±0.22	224.59±1.93

Table 4. Estimated molecular weight, protein and peptide concentration of the nine SEC HPLC fractions, Mean±SD, n=3.

Fractions 7 and 8 were the only fractions to demonstrate a dose-dependent antiproliferative effects on MCF-7 cells with the most potent effect exhibited with fraction 7 (Figure 2).

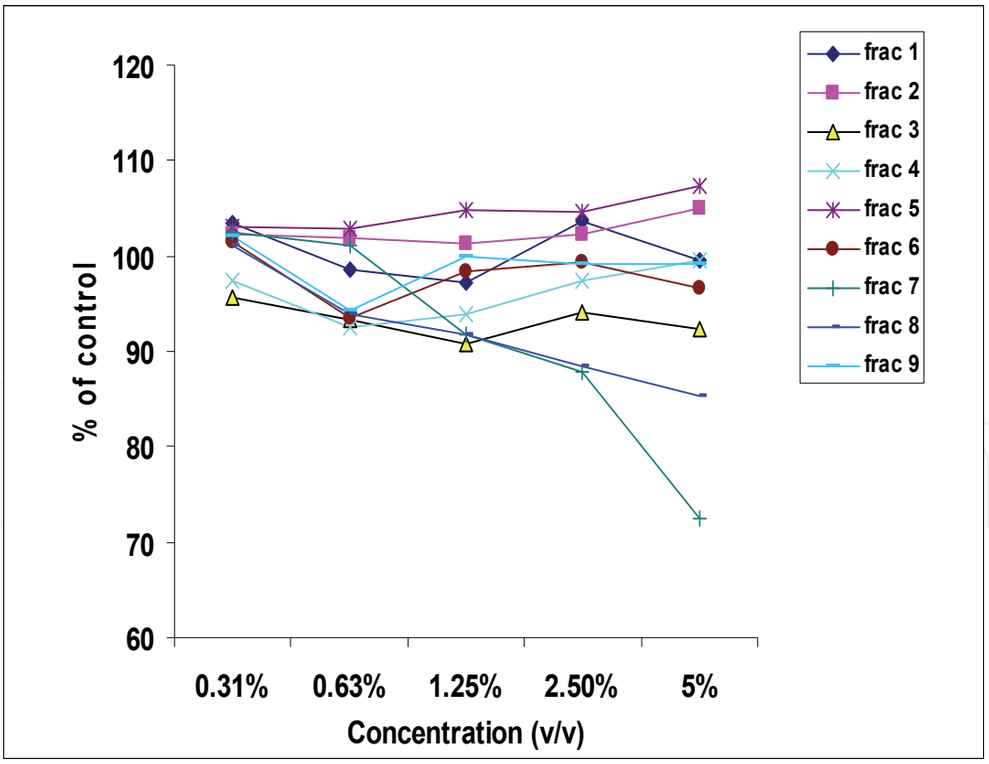


Fig. 2. Antiproliferative effects of 9 SEC-HPLC fractions on MCF-7 cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean±SD, n=4.

Fraction 7 was also tested with HMEC cells, and no antiproliferative effects were observed (Figure 3). Fraction 7 was further analyzed with RP-HPLC, which showed the presence of about 11 peaks in Fraction 7 (Figure 4). SEC-HPLC Fraction 7 was also analyzed via MALDI-TOF, which indicated that the peak masses ranged from 659 to 2074 Da. (Figure 5).

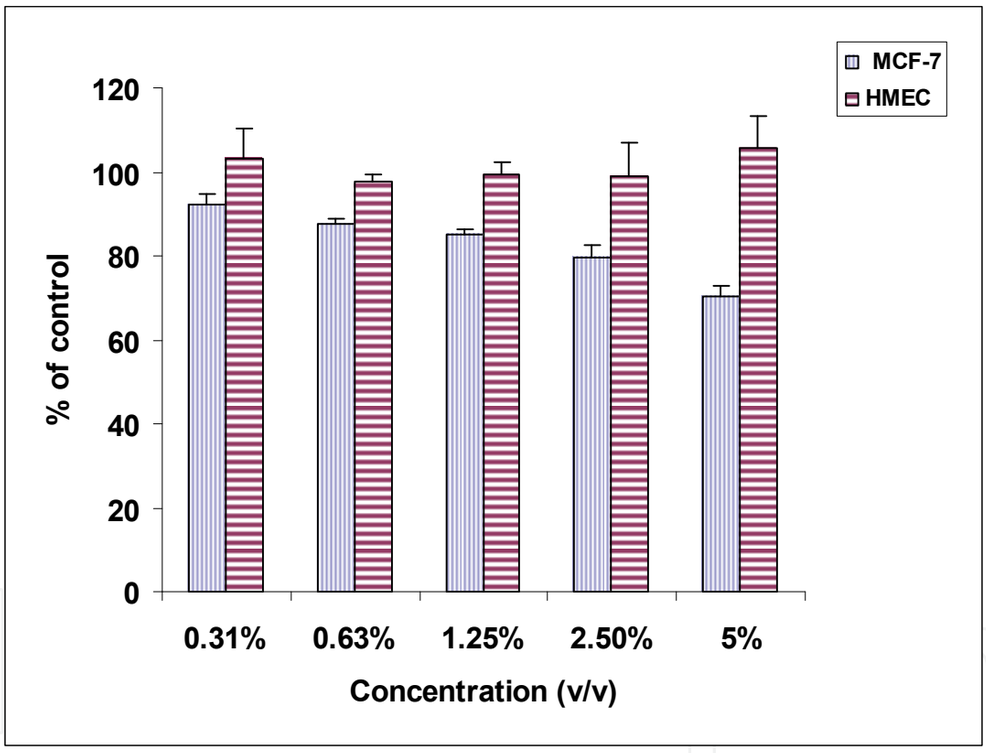


Fig. 3. Antiproliferative effects of SEC-HPLC Fraction 7 on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean+SD, n=4.

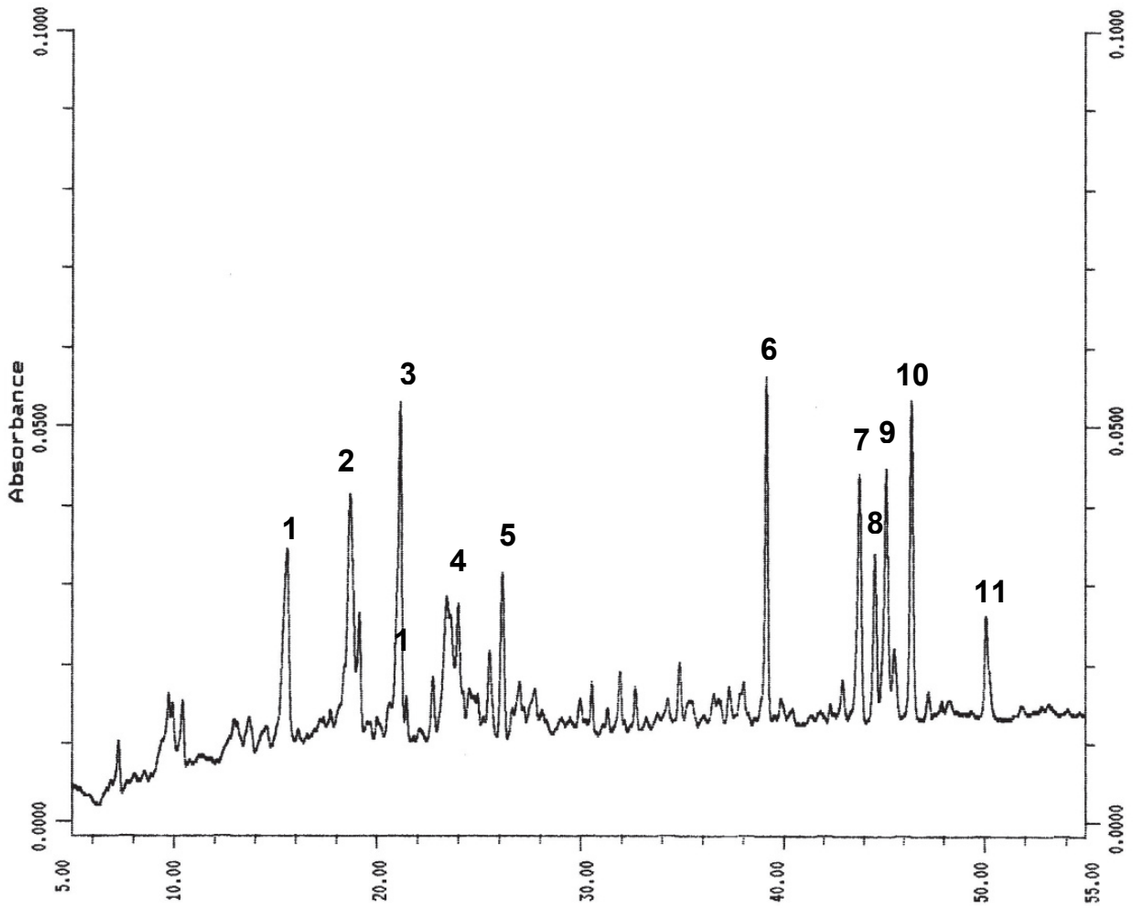


Fig. 4. Elution profile of SEC-HPLC Fraction 7 of kefir mother culture using RP-HPLC. A Prosphere 300 C4, 5 m, 250 mm x 4.6 mm column was used. After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 ml per min, the fractions were eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm and channel B 280 nm. The retention times of the peaks 1 to 11 are 15.4, 18.6, 21.1, 23.3, 26.1, 39.1, 43.7, 44.5, 45.2, 46.3, and 50.1 min, respectively.

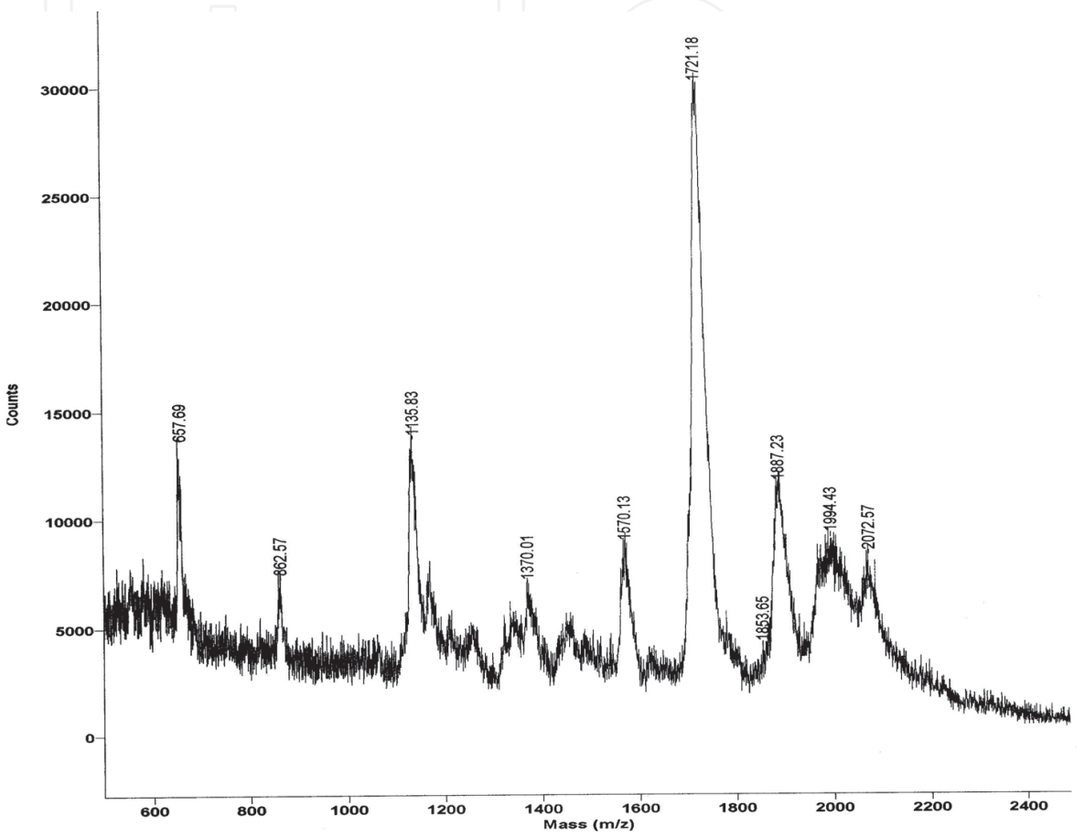


Fig. 5. Mass spectrogram of SEC-HPLC Fraction 7 of kefir mother culture analyzed by a MALDI-TOF mass spectrometer with a laser at 337 nm and an acceleration voltage of 20.000 V.

A preparative C4 column was utilized to obtain sufficient amounts of mother culture kefir fractions of MWCO less than 3000 Da for more extensive structure analysis and cell culture studies. The average nitrogen content of the lyophilized filtrate of MWCO less than 3000 Da was 0.58%. Five g of the lyophilized MWCO less than 3000 Da fractions was dissolved in 20 ml of water. Ten milliliters of reconstituted solution were loaded on the column, and 100 fractions were collected in 1 min intervals (Figure 6).

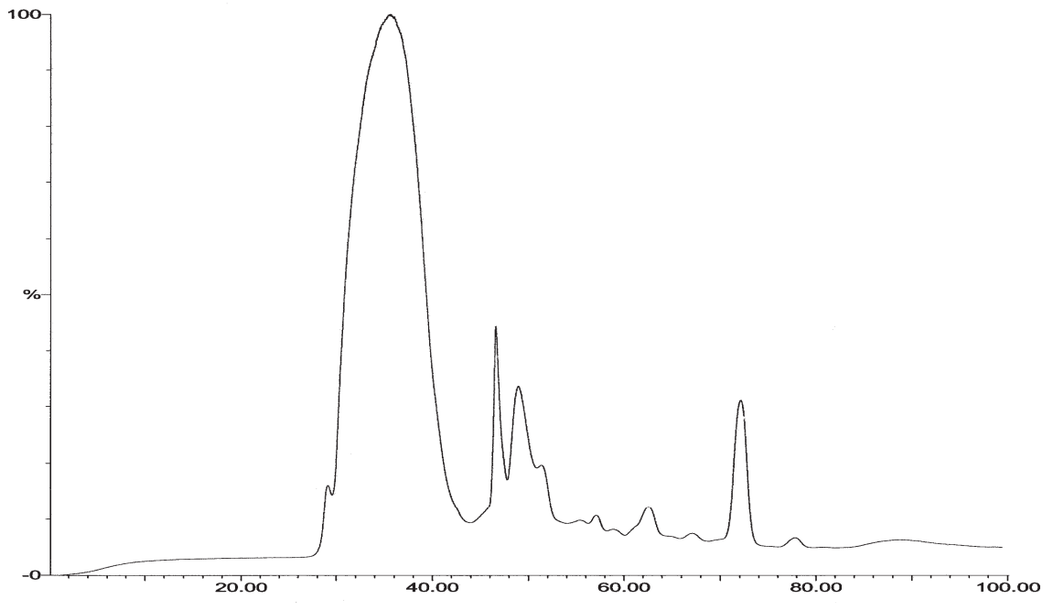


Fig. 6. Elution profile of MWCO less than 3000 Da fraction of kefir mother culture extract analyzed by preparative RP-HPLC. Appropriate amount of samples were loaded on a C4 preparative column (300 Å, 5 m, 300 mm x 50 mm) (Vydac Company, Herperia, CA) and separated with Waters Delta Prep 4000 HPLC system. After the column was equilibrated with buffer A at a flow rate of 13 ml, the fractions were eluted with a linear gradient of Buffer A (0.6% acetic acid in water) and Buffer B (0.6% acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B. The absorbance was measured at 210 nm. A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals.

The peptide concentration of each preparative HPLC fraction is shown in Figure 7, Fractions 28 to 58, 62 to 64, and 72 to 74 were screened for antiproliferative effects on MCF-7 cells with three concentrations of 0.4%, 2% and 10% (Figure 8).

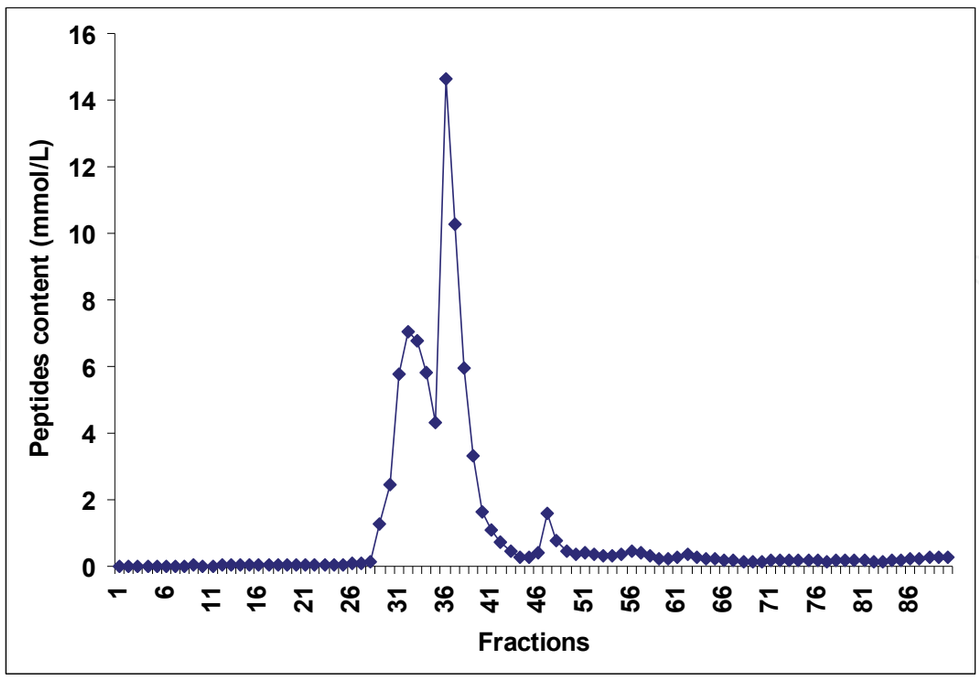


Fig. 7. Peptide concentrations in preparative RP-HPLC fractions. One hundred fractions obtained from preparative RP-HPLC were freeze-dried and then reconstituted with nanopure water to certain concentration. Peptide concentrations were determined in triplicates by OPA method. Mean values were plotted.

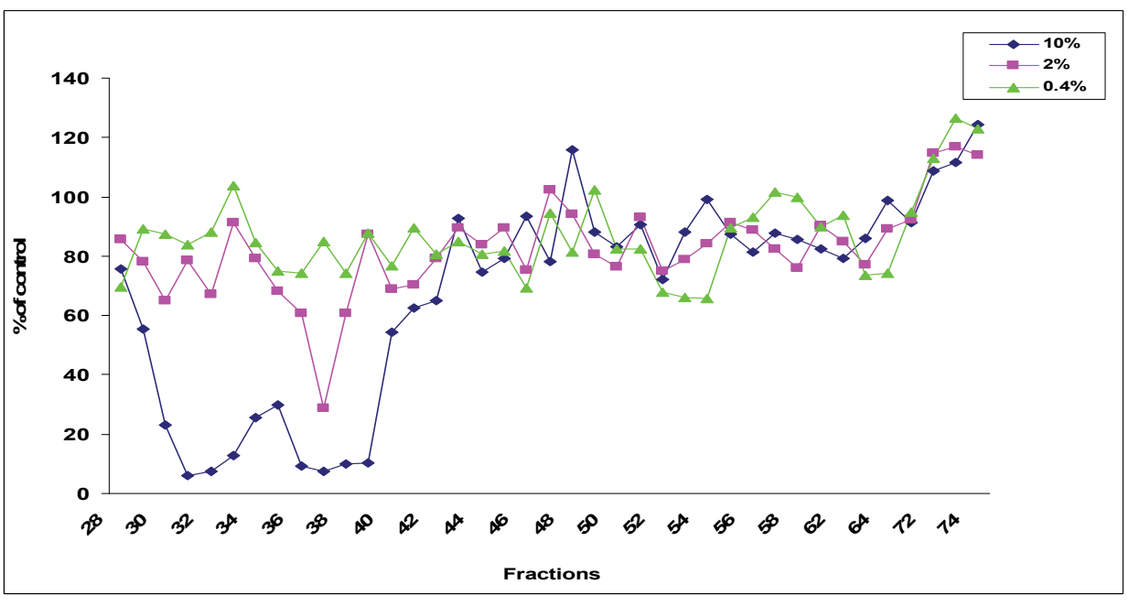


Fig. 8. Antiproliferative effects of fractions of preparative RP-HPLC on MCF-7 cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Each fraction was diluted at three concentrations and was run in quadruplicate. Mean values were plotted.

Fractions 29 to 41, 62 were found to have antiproliferative effects on MCF-7 cells. These fractions were further screened for antiproliferative effects with serial dilution. A dose dependent antiproliferative effect was observed when MCF-7 cells were treated with fractions 29, 30, 34, and 37, while not on HMEC cells (Figure 9).

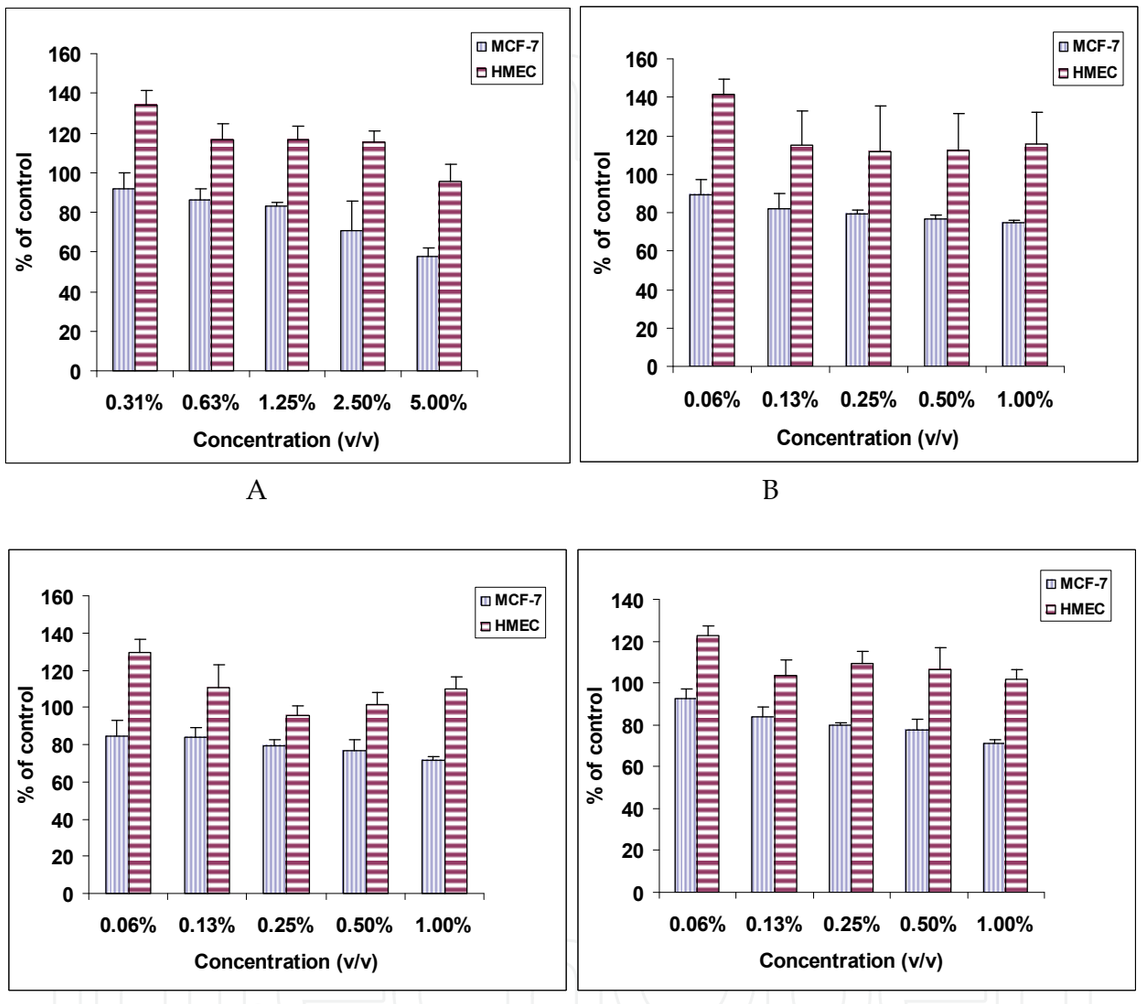


Fig. 9. Dose effect of preparative RP-HPLC Fraction 29 (A), 30 (B), 34 (C), and 37 (D) on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean±SD, n=4.

Tamoxifen is a commonly used medication for breast cancer patients. In this study, only the dosage above 0.2 M/l showed antiproliferative effects on MCF-7 cells. The effective dose reported here was higher than the value reported by Doisneau-Sixou *et al.* (2003). RP-HPLC Fraction 30 of kefir mother culture extract significantly increased MCF-7 cell susceptibility to tamoxifen (Figure 10). A dose dependent antiproliferative effect was also observed.

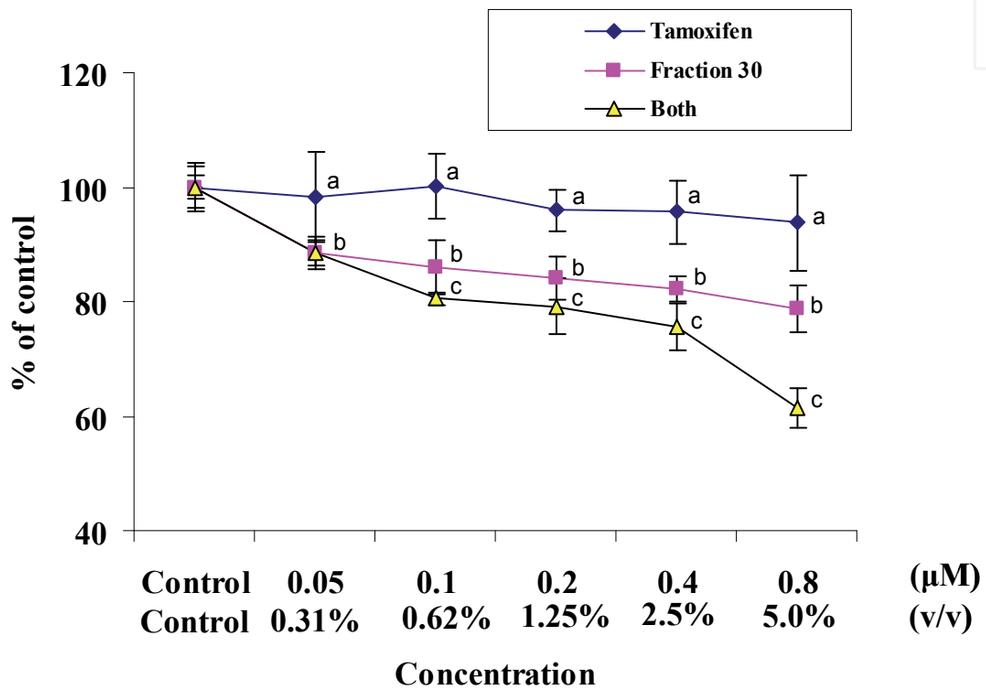


Fig. 10. Antiproliferative effect of RP-HPLC Fraction 30 of kefir alone or in combination with tamoxifen on MCF-7 cells. Means±SD (n=4) with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.

Electrospray mass spectrometry was used to determine the molecular weight profile of the active Fractions 29, 30, 34, and 37. Figure 11 is the ESI-mass spectrum of HPLC fraction 30. The spectrum represents a typical molecular ion profile of the active HPLC fractions (29, 30, 34 and 37). The similarity in the molecular ion profile of the active fractions indicated that the active component(s) is spread over HPLC Fractions 30 to 37. The spectrum showed mainly singly charged molecular ions with m/z ratios ranging from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

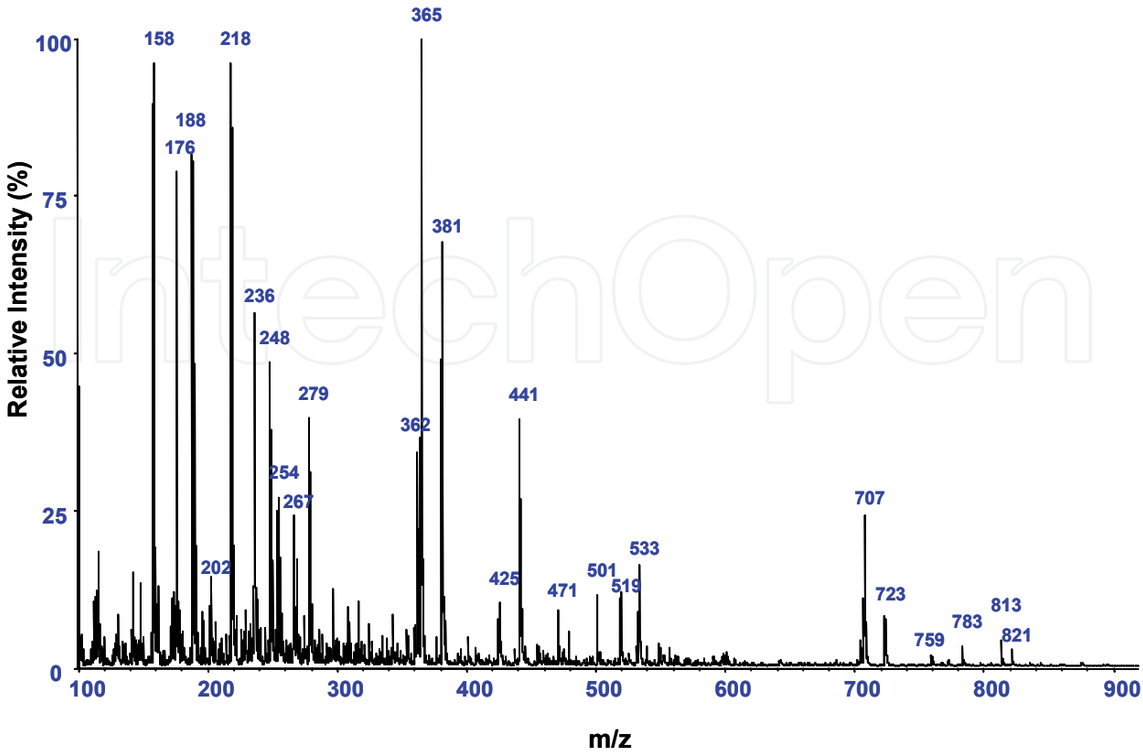
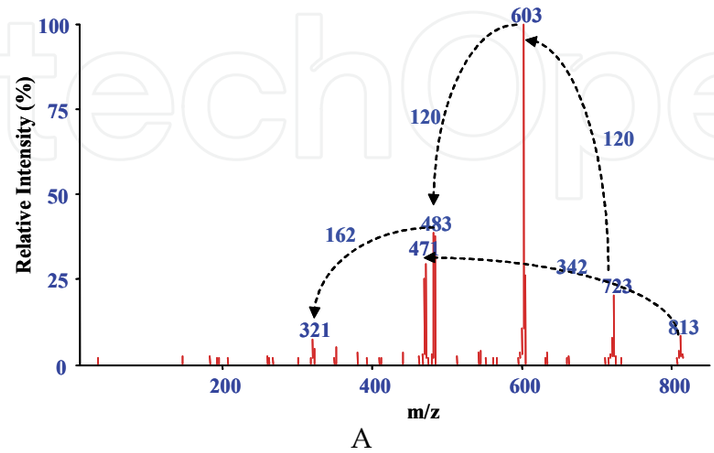


Fig. 11. Mass spectrum of HPLC Fraction 30 done by electrospray mass spectrometry. The mainly singly charged molecular ions with m/z ratios range from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

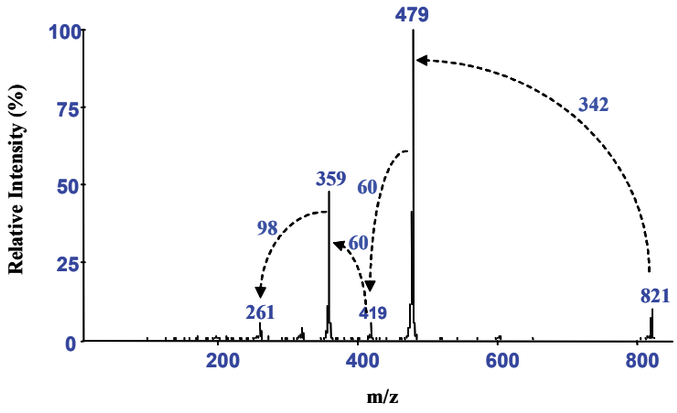
To characterize the composition and chemical structure of the active components in the HPLC fractions, molecular ions ranging from m/z 300 to 900 were subjected to collision ion dissociation (CID) tandem mass spectrometry. Figure 12A, showed the CID spectrum of the molecular ion at m/z 821. Fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 mass units, which corresponds to the loss of a lactose moiety. It also produced a series of neutral loses of 60 mass units, which correspond to the loss of acetic acid moieties. The neutral loss of 60 atomic mass units may also be derived from sugar cleavage products (Figure 13). The CID spectrum of the molecular ion at m/z 813 (Figure 12B) also produced a neutral loss of 342 (lactose) and neutral losses of 120 atomic mass units, which may be derived from fragmentation of lactose (Figure 13). A neutral loss of 162, which corresponds to the loss of a hexose moiety (glucose or galactose), is also observed. This was also observed at m/z 759. The fragmentation patterns of the molecular ions at m/z 821 and 813 indicate that they have similar structural features. The molecular ions with m/z 707 (Figure 12E) and 723 (Figure 12D) represent sodium and potassium ion dimmers of lactose, respectively. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively (Figure 14).

Another interesting feature about the fragmentation pattern of with m/z ranging from 707 to 823 is that they fragmented into common fragment ions at m/z 261 and 98. Further isolation and fragmentation (MS-MS) of the ion at m/z 261 showed that its major product ion is the ion at m/z 98. Further isolation and fragmentation of the ion at m/z 98 produced

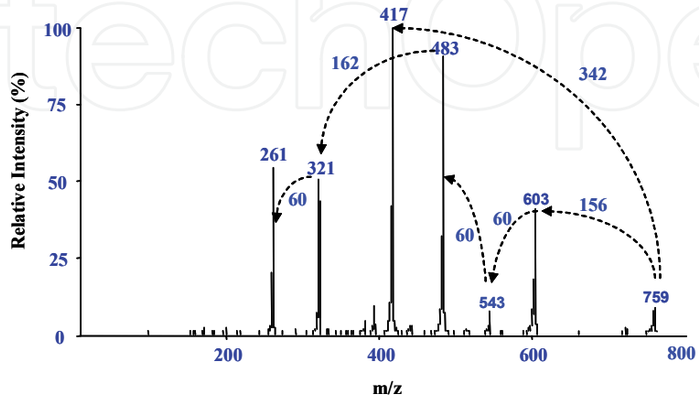
two ions at m/z 54 and 39. The tandem mass spectrometric analysis of the molecular ion of the active HPLC fraction show that most of the peaks produced a stable product ion at m/z 98, but the ion at 98 could not be ascribed to known structure peptide. Analysis of the fragmentation pattern of the molecular ion at m/z 759 (Figure 12C), however, revealed that the molecular ion could be derived from the interaction between serine and lactose.



A

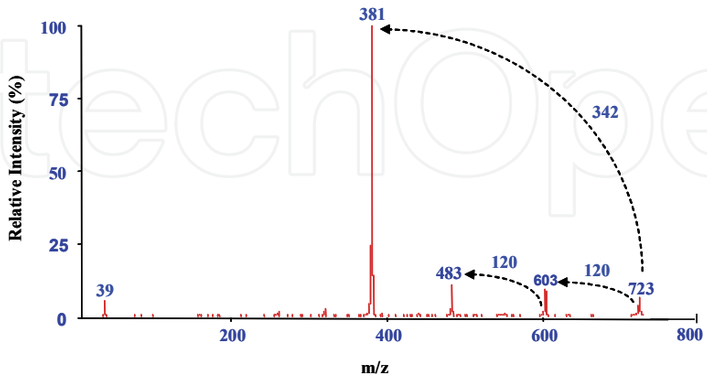


B

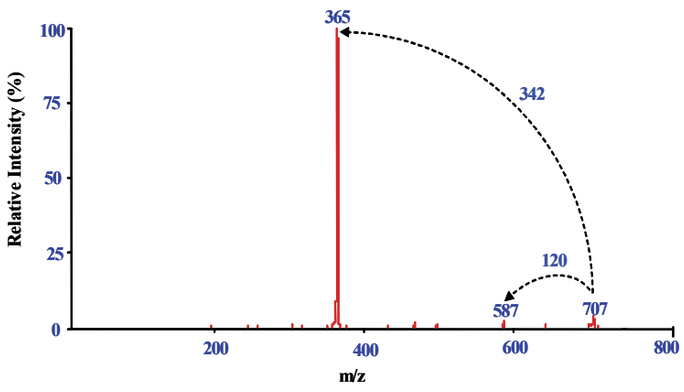


C

Fig. 12. (Continued)



D



E

Fig. 12. Spectrum of molecular ion at m/z = 821 (A), m/z=813 (B), m/z= 759 (C), m/z=723 (D), and m/z=707 (E) done by collision ion dissociation (CID) tandem mass spectrometry. As indicated in Figure A, fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 and 60 mass units, which corresponds to the loss of a lactose moiety and acetic acid moiety respectively. There is also a neutral loss of 98, which could not be ascribed to a known structure. Ion at m/z =261 was also observed. Fragmentation of the molecular ion at m/z 813 produced a neutral loss of 342 and 120 mass units, which might be derived from lactose and hexose moieties (glucose or galactose) respectively. Fragmentation of the molecular ion at m/z 759 produced a neutral loss of 342 and serial loss of 60 mass units. Fragmentation of the molecular ion at m/z=723 produced neutral losses of 342 and 120 atomic mass unit. The main fragment is the ion at m/z 365 with the neutral loss of 342 (lactose) from m/z 707.

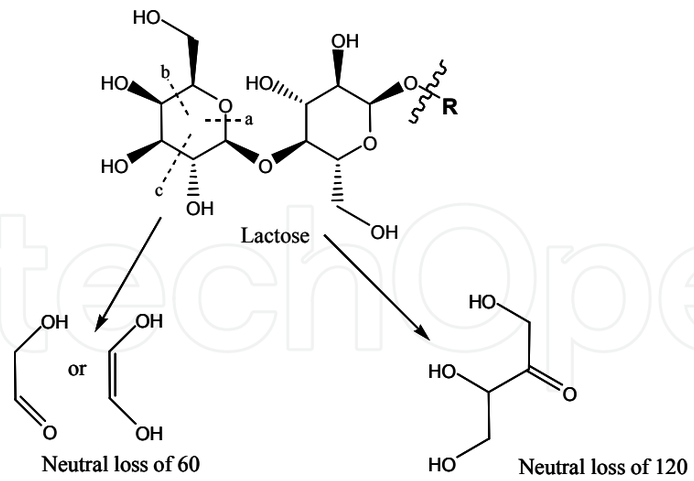


Fig. 13. Scheme 1: Generation of neutral loss of 60 and 120 atomic mass unit from fragmentation of lactose. Fragmentation at "a" and "b" will generate a neutral loss of 60 and fragmentation at "a" and "c" will generate a neutral loss of 120 atomic mass unit. They can be derived from fragmentation of acetic acid and lactose.

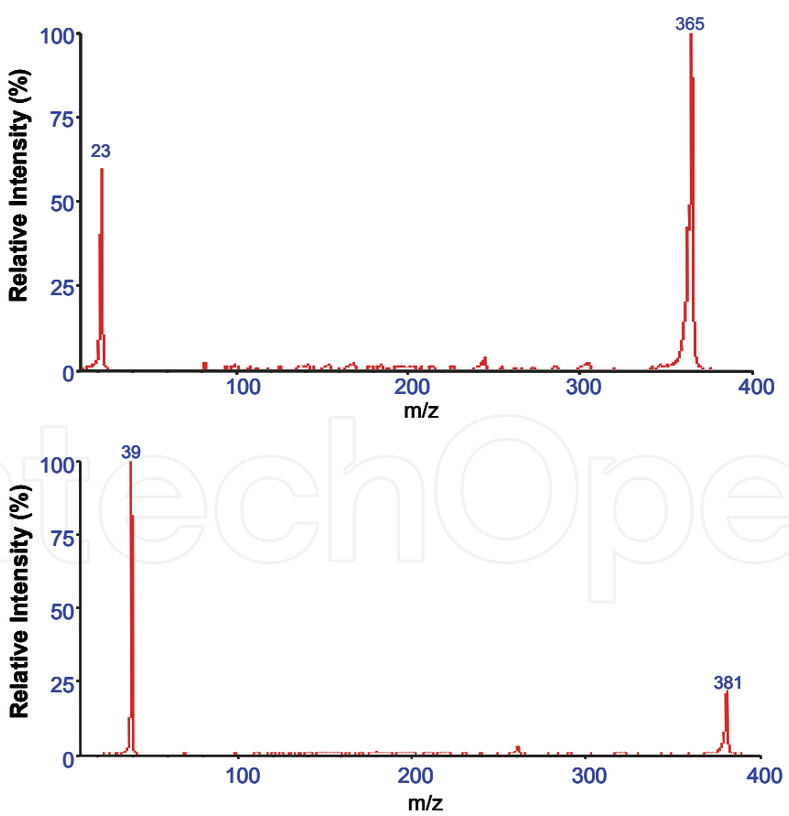


Fig. 14. Spectrum of molecular ion at m/z 365 and 381 done by collision ion dissociation (CID) tandem mass spectrometry. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively.

A possible scheme for the generation of the ion at m/z 759 is presented in Scheme 2 (Figure 15). The proposed structure agrees with the fragmentation pattern. This interaction may be due to chemical reactions. The interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases. The active fraction likely still contains a mixture of chemicals as suggested by the results of RP-HPLC (Figure 16). The exact molecular structure of these compounds is not known. However, results of the extensive MS analysis suggested that the compounds are possibly complex polymers that have lactose as the backbone structure. The proposed structure shown in Scheme 2 is a product with two lactose units linked by a serine. A more complex molecule with linkage with acetic acid is also possible.

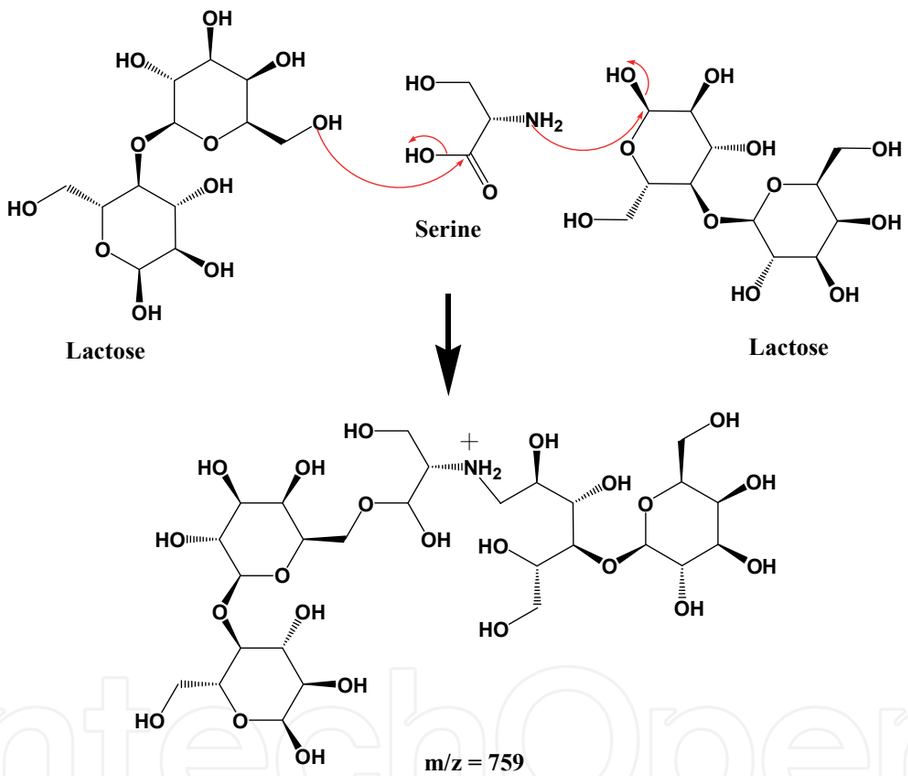


Fig. 15. Scheme 2: A possible interaction for the generation of the ion at m/z 759. As shown in the scheme, the interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases.

The isolated bioactive fraction showed antiproliferative effects on cancer cells while not on normal cells. As indicated in the RP-HPLC chromatograph, the isolated fractions such as Fraction 30 are likely composed of multiple components that may be acting either singly or synergistically to exert the antiproliferative effects. Although the presence of peptides was suggested in the fractions as determined by the OPA methodology, the MS data did not

support the presence of known peptide structures. The failure to identify any known peptides is likely due to the limitation of the OPA method, which can only detect the presence of primary amines and thus the primary amines in peptides cannot be differentiated from other amine containing compounds.

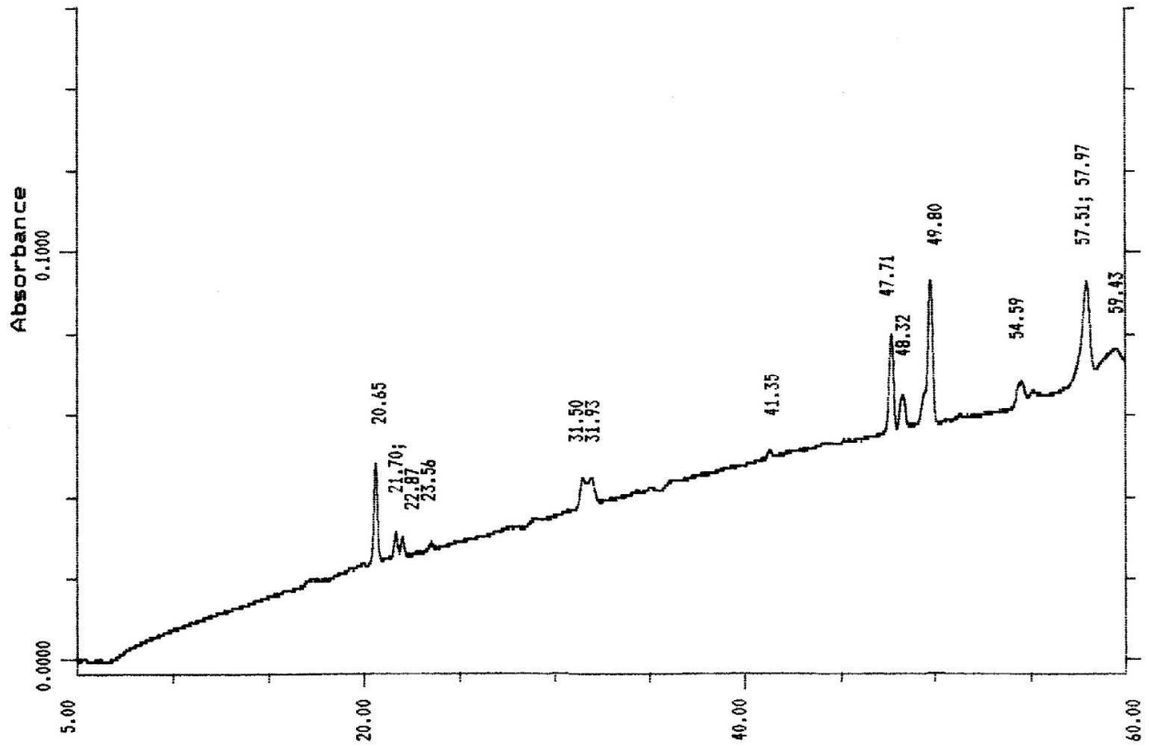


Fig. 16. Chromatograph of preparative HPLC Fraction 30 done by RP-HPLC. 50 l of reconstituted preparative HPLC Fraction 30 which showed antiproliferative effect on MCF-7 cells was injected into a Prosphere 300 C4 column (5 m, 250 mm x 4.6 mm) (Alltech Associate, Inc. Deerfield, IL 60015, USA) on Beckman HPLC System and eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm.

4. Conclusion

Due to the complex mixture of components in the specific fractions and the whole kefir extracts, the antiproliferative role of specific components such as proteins, peptides, organic acids and some small molecules, such as free amino acids, oligosaccharides and their interactions has been unclear. One of the main components of the bioactive antiproliferative kefir fractions is composed of lactose and acetic acid, possibly an end product of fermentation of the milk protein and sugar by the kefir bacteria and yeast. The lactose polymer suggests that the active component may be fragments of previously isolated polysaccharides termed as kefirans (Kooiman, 1968). Kefiran is a water-soluble polysaccharide consisted of approximately equal proportions of D-glucose and D-galactose (Kooiman, 1968; Micheli *et al.*, 1999). Kefiran has been reported to have antitumor activity

(Shiomi *et al*., 1982; Murofushi *et al*., 1983). It was also shown to be one of the substances having few or no side effect(s) in functioning to retard tumor growth in vivo.

It is noteworthy that one of the proposed novel bioactive structures contains serine and glucose or galactose; hence, it is conceivable that this molecule could be a sphingolipid compound. The ceramide component of sphingolipids is derived from serine and sphingolipids such as cerebrosides, which contain either a glucose or galactose. Sphingolipids such as gangliosides are sphingosine compounds that contain several glucose or galactose units. Also, lower molecular weight ceramide moieties have been detected in dairy gangliosides (Colarow *et al*., 2003) and cultured dairy products have been shown to be a rich source of gangliosides (Kathleen *et al*., 2000). Ceramide is derived from sphingomyelin (SpM) and can act as an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, and other cytokines. Ceramide has also been implicated in the acquired drug resistance that often characterizes breast cancer cells (Liu *et al*., 1999). C2 and C6 ceramides are cell permeable ceramide analogs that have been shown to induce cell apoptosis (Fillet *et al*., 2003). Osada *et al.* (1993) demonstrated that kefir contains an active substance, which enhances IFN-beta secretion of a human osteosarcoma line MG-63 treated with a chemical inducer, poly I: poly C. The active substance in the fermented milk was identified to be SpM. SpM from fermented milk (F-SpM) was a mixture of four molecular species of SpMs having C21-, C22-, C23- and C24-fatty acids. F-SpM enhanced the IFN secretion 14 times; SpMs from other food sources also enhanced IFN secretion but more moderately (2-3 times). We have observed a synergistic effect when MCF-7 cells were treated with ceramide analogs in the presence of extract of mother culture (data not shown). The putative sphingolipid component(s) in kefir that we have detected may have acted synergistically with the ceramide analogs leading to apoptosis of MCF-7 cells. In support of this concept, SpM has been shown to enhance the ceramide formation and ceramide-induced apoptosis in concert with the chemotherapeutic agent, gemcitabine, in human pancreatic cancer cells (Modrak *et al*., 2004). Modification of ceramide metabolism also increases MCF-7 cells sensitivity to cytotoxics (Lucci *et al.*, 1999).

In summary, we have isolated the bioactive components from kefir mother culture that show antitumor cell proliferative effects. The MS data indicate that the isolated compounds are likely SpM complexes such as gangliosides that might have anti-tumor activities that are similar to that of ceramide. The biological functions of these ceramide-like compounds warrant further studies. The appearance of multiple components in the isolated bioactive antitumor fractions suggests the possibility that such components may be acting both independently and synergistically to exert the antiproliferative effects. Instead of searching for single compound in kefir, a mixture or a combination of components may be the best approach to have the highest potency for antitumor properties. The potential of using the kefir extract as co-drug for chemotherapy should be explored. In that regard, RP-HPLC Fraction 30 increased significantly the susceptibility of MCF-7 cells to tamoxifen, a commonly used anti-breast cancer drug. These results thus provide the rationale for future research to explore the potential of using kefir extracts as co-drugs for breast cancer chemotherapy and/or a functional food used for prevention of breast cancer.

5. Acknowledgments

This study was supported in part by grants from Liberty Brand Products, Inc. and a Natural Science and Engineering Research of Canada grant to S.K. and H.M.C. The authors thank

the contribution of Dr. Kobi Yeboah for the mass spectrometry analysis. S.K. owns shares in KCLM Research in Nutrition Inc., which holds an exclusive option to license the intellectual property rights to the anticancer properties of kefir and kefir extracts.

6. References

Biffi, A.; Coradini, D., Larsen, R., Riva, L. & Di Fronzo, G. (1997). Antiproliferative Effect of Fermented Milk on the Growth of a Human Breast Cancer Cell Line. *Nutrition and Cancer*, Vol.28, No.1, (January 1997), pp. 93-99, ISSN 0163-5581

Bradford, M.M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry,* Vol.72, No. 1-2, (May 1976), pp. 248–254, ISSN 0003-2697

Cevikbas, A.; Yemni, E., Ezzedenn, F.W. & Yardimici, T. (1994). Antitumoral Antibacterial and Antifungal Activities of Kefir and Kefir Grain. *Phytotherapy Research,* Vol.8, No.2, (March 1993) pp. 78-82, ISSN 0951-418X

Chen, C.; Chan, H.M., & Kubow, S. (2007). Kefir Extracts Suppress In Vitro Proliferation of Estrogen-Dependent Human Breast Cancer Cells But Not Normal Mammary Epithelial Cells. *Journal of Medicinal Food,* Vol.10, No.3, (September 2007), pp. 416-422, ISSN 1096-620X

Church, F.C.; Swaisgood, H.E., Porter, D.H. & Catinani, G.L. (1983). Spectrophotometric Assay Using *O*-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolation Milk Proteins. *Journal of Dairy Science,* Vol.66, No.6, (June 1983), pp. 1219-1227

Colarow, L.; Turini, M., Teneberg, S. & Berger, A. (2003). Characterization and Biological Activity of Gangliosides in Buffalo Milk. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, Vol.1631, No.1, (February 2003), pp. 94-106, ISSN 0006-3002

Dillehay, D.L.; Webb, S.J., Schmelz, E.-M. & Merrill, A.H.Jr. (1994). Dietary Sphingomyelin Inhibits 1,2-Dimethylhydrazine-Induced Colon Cancer in CF1 Mice. *Journal of Nutrition,* Vol.124, No.5, (May 1994), pp. 615–620, ISSN 0022-3166

Doisneau-Sixou, S.F.; Cestac, P., Faye, J.C., Favre, G. & Sutherland, R. (2003). Additive Effects of Tamoxifen and the Farnesyl Transferase Inhibitor FTI-277 on Inhibition of MCF-7 Breast Cancer Cell-Cycle Progression. *International Journal of Cancer,* Vol.106, No.5, (September 2003), pp. 789-798, ISSN 0020-7136

Fillet, M.; Bentires-Alj, M., Deregowski, V., Greimers, R., Gielen, J., Piette, J., Bours, V. & Merville, M.P. (2003). Mechanisms Involved in Exogenous C2- and C6-Ceramide-Induced Cancer Cell Toxicity. *Biochemical Pharmacology,* Vol.65, No.10, (May 2003), pp. 1633-1642, ISSN 0006-2952

Furukawa, N.; Matsuoka, A., Takahashi, T. & Yamanaka, Y. (2000). Anti-Metastatic Effect of Kefir Grain Components on Lewis Lung Carcinoma and Highly Metastatic B16 Melanoma in Mice. *Journal of Agriculture Science Tokyo Nogyo Daigaku,* Vol.45, No.1, (January 2000), pp. 62-70, ISSN 0375-9202

Garrote, G.L.; Abraham, A.G. & Antoni, G.L. (2000). Inhibitory Power of Kefir: The Role of Organic Acids. *Journal of Food Protection,* Vol.63, No.3, (March 2000), pp. 364-369, ISSN 0362-028X

Guzel-Seydim, Z.B.; Seydim, A.C. & Greene, A.K. (2003). Comparison of Amino Acid Profiles of Milk, Yogurt and Turkish Kefir. *Milchwissenschaft,* Vol.58, No.3 (March 2003), pp. 158-160, ISSN 0026-3788

Kathleen, H.; Moore A., Ettinger, C. & Melvin, T.Y. (2000). Variation in Ganglioside Content of Bovine Dairy Products. *Journal of Food Composition and Analysis,* Vol.13, No.5 (March 2000), pp. 783-790, ISSN 0889-1575

Kooiman, P. (1968). The Chemical Structure of Kefiran, the Water-Soluble Polysaccharide of the Kefir Grain. *Carbohydrate Research,* Vol.7, No.2, (June 1968), pp. 200-211

Liu, Y.-Y.; Han, T.-Y., Giuliano, A.E., Ichikawa, S., Hirabayashi, Y. & Cabot, M.C. (1999). Glycosylation of Ceramide Potentiates Cellular Resistance to Tumor Necrosis Factor-Alpha-Induced Apoptosis. *Experimental Cell Research,* Vol.252, No.2 (November 1999), pp. 464-470, ISSN 0014-4827

Lucci, A.; Han, T.Y., Liu, Y.Y., Giuliano, A.E. & Cabot, M.C. (1999). Modification of Ceramide Metabolism Increases Cancer Sensitivity to Cytotoxics. *International Journal of Oncology,* Vol.15, No. 3, (September 1999), pp. 541-546, ISSN 1019-6439

Mesner, P.W. Jr.; Budihardjo, I.I. & Kaufmann, S.H. (1997). Chemotherapy-Induced Apoptosis. *Advances in Pharmacology,* Vol.41, pp. 461-499

Micheli, L.; Uccelletti, D., Paleschi, C. & Crescenzi, V. (1999). Isolation and Characterization of a Lactobacillus Strain Producing the Exopolysacchsride Kefiran. *Applied Microbiology and Biotechnology,* Vol.34, No.1 (December 1999), pp. 137-143, ISSN 0175-7598

Modrak, D.E.; Cardillo, T.M., Newsome, G.A., Goldenberg, D.M. & Gold, D.V. (2004). Synergistic Interaction Between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer. *Cancer Research,* Vol. 64, No.22, (November 2004), pp. 8405-8410, ISSN 0008-5472

Murofushi, M.; Shiomi, M. & Aibara, K. (1983). Effect of Orally Administered Polysaccharide from Kefir Grain on Delayed-Type Hypersensitivity and Tumor Growth in Mice. *Japanese Journal of Medical Science and Biology,* Vol.36, No.1 (February 1983), pp. 49-53, ISSN 0021-5112

Osada, K.; Nagira, K., Teruya, K., Tachibana, H., Shirahata, S. & Murakami, H. (1993). Enhancement of Interferon-Beta Production with Sphingomyelin from Fermented Milk. *Biotherapy,* Vol.7, No.2, (February 1993), pp. 115-123, ISSN 0921-299X

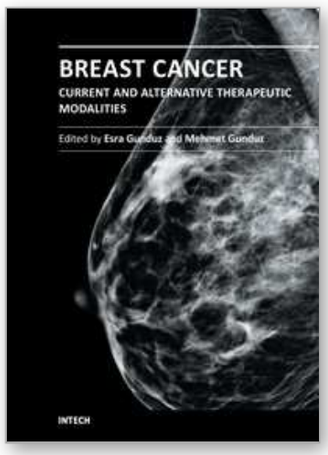
Parvez, S.; Malik, K.A., Ah, Kang. S. & Kim, H.Y. (2006). Probiotics and Their Fermented Food Products are Beneficial for Health. Journal of Applied Microbiology, Vol.100, No.6, (June 2006), pp. 1171-1185, ISSN 1364-507

Ronco, A.L., Stefani, E. & Dattoli, R. (2002). Dairy Foods and Risk of Breast Cancer: A Case-Control Study in Montevideo, Uruguay. *European Journal of Cancer Prevention*. Vol.11, No.5, (October 2002), pp. 457-463, ISSN 0959-8278

Schonberg, S. & Kordan H.E. (1995). The Inhibitory Effects of Conjugated Dienoic Derivatives (CLA) of Linoleic Acid on the Growth of Human Tumor Cell Lines is in Part Due to Increased Lipid Peroxidation. *Anticancer research,* Vol.15, No.4, (July-August 1995), pp. 1241-1246, ISSN 0250-7005

Shiomi, M.; Sasaki, K., Murofushi, M. & Aibara, K. (1982). Antitumor Activity in Mice of Orally Administered Polysaccharide from Kefir Grain. *Japanese Journal of Medical Science and Biology,* Vol.35, No.2 (April 1982), pp. 75-80, ISSN 0021-5112

Svensson, M.; Sabharwal, H., Mossberg, A.K., Lipniunas, P., Leffler, H., Svanborg, C. & Linse, S. (1999). Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *Journal of Biological Chemistry,* Vol.274, No.10 (March 1999), pp. 6388-6396, ISSN 0021-9258



Breast Cancer - Current and Alternative Therapeutic Modalities
Edited by Prof. Esra Gunduz

ISBN 978-953-307-776-5
Hard cover, 540 pages
Publisher InTech
Published online 09, November, 2011
Published in print edition November, 2011

Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Chujian Chen, Hing Man Chan and Stan Kubow (2011). Fractionation and Characterization of Bioactive Components in Kefir Mother Culture that Inhibit Proliferation of Cultured MCF-7 Human Breast-Cancer Cells, Breast Cancer - Current and Alternative Therapeutic Modalities, Prof. Esra Gunduz (Ed.), ISBN: 978-953-307-776-5, InTech, Available from: http://www.intechopen.com/books/breast-cancer-current-and-alternative-therapeutic-modalities/fractionation-and-characterization-of-bioactive-components-in-kefir-mother-culture-that-inhibit-prol

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

© 2011 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the Creative Commons Attribution 3.0 License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen