A Master's Thesis

Changes in phenolic compounds, antioxidant and antiproliferative activities of blueberry during *Lactobacillus plantarum* fermentation

Ji-yeon Ryu

School of Biomaterials Science and Technology

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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지도교수 김소미

류지연

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심사위원장 이 종은

위 원 조 윤지

위 원 김 소 미

제주대학교 대학원

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Ji-yeon Ryu

(Supervised by professor Somi Kim Cho)

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This thesis has been examined and approved.

Pang Gyu Lee

Moonjae Cho

Somi Kim Cho

2018. 2

School of Biomaterials Science and Technology

Graduate School

Jeju National University

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ABSTRACT

Blueberries are a rich source of dietary bioactive compounds, including phenolic compounds, flavonol, flavanol, tannin, vitamin C and especially anthocyanin. Fermentation has been the most widely used in the food industry and a variety of food cultures. Among them, lactic acid fermentation is known to impart unique flavor and taste, excellent preservation and physiological functions to food. In this study, changes in phenolic compounds, antioxidant and antiproliferative activities of blueberry during *Lactobacillus plantarum* fermentation were investigated. The bacterial cell viability and total titratable acidity were increased during fermentation while pH value and total soluble solid were decreased. Fermented blueberry showed increases in total polyphenol and flavonoid content, DPPH-, ABTS-, Alkyl- radical scavenging activities and SOD-like activity. The antiproliferative activities of fermented blueberry were demonstrated in human cervical carcinoma HeLa cells using MTT assay, Hoechst 33342 staining, annexin V/PI staining and cell cycle analysis. There was an increase of the apoptotic body formation in the cells treated with fermented blueberry while no apoptotic body formation was detected in the cells treated with non-fermented blueberry. Annexin V/PI double staining and cell cycle analysis showed that fermented blueberry induces apoptosis in HeLa cells. In addition, GC-MS and HPLC analysis showed that lactic acid, catechol, protocatechuic acid, chlorogenic acid, and ferulic acid were increased during fermentation. In conclusion, our results suggest that antioxidant and anticancer effects

are increased in HeLa cells with fermentation of blueberries, which indicates that blueberries are a food material that can be applied to the functional food industry.

요약문

블루베리는 페놀 화합물, 플라보놀, 플라바놀, 탄닌, 비타민 C 등과 같은 활성 물질을 함유하고 있으며, 특히 안토시아닌이 풍부하다고 알려져 있다. 발효는 식품 산업과 문화에서 널리 사용되어 왔으며, 그 중 유산균 발효는 특유의 향과 맛을 가지며, 생리 활성을 부여하는 것으로 알려져 있다. 본 연구에서는 *Lactobacillus plantarum*을 이용하여 블루베리를 발효하고, 발효 기간 동안의 페놀 화합물, 항산화 및 항증식 활성의 변화에 대해 연구하였다. 유산균의 생균수와 총산도는 발효가 진행됨에 따라 증가하였으며, pH와 총당도는 감소하였다. 발효한 블루베리의 경우 총 폴리페놀과 플라보노이드 함량, DPPH-, ABTS-, Alkyl - 라디칼 소거능, SOD 유사 활성이 증가되었다. 인간 자궁경부암 HeLa 세포에 발효한 블루베리 추출물을 처리했을 때 apoptotic bodies 형성이 증가한 반면, 발효하지 않은 블루베리 추출물을 처리했을 때는 apoptotic bodies가 관찰되지 않았다. Annexin V/PI staining과 cell cycle analysis를 통해 발효한 블루베리가 HeLa 세포에서 세포사멸을 유도함을 확인하였다. 또한, GC-MS 와 HPLC 분석을 통해 발효에 의해 lactic acid, catechol, protocatechuic acid, chlorogenic acid, ferulic acid와 rutin이 증가하는 것으로 확인하였다. 본 연구 결과를 통해 블루베리의 유산균 발효에 의해 항산화효능과 항암 효능이 증가하는 것을 확인하였으며, 이러한 결과를 통해 유산균 발효 블루베리가 기능성 식품 산업에 적용 가능할 것으로 보여진다.

1. Introduction

Cervical cancer is the second most common malignant disease in women worldwide. Despite the continuous development of therapeutic agents, the survival rate of the patients is still low and the mortality rate is high. The cause of cervical cancer is mainly caused by HPV infection, and chemotherapy, surgery, and radiotherapy are being used as therapeutic modalities. However, side effects of anticancer agents such as cisplatin have been shown (19). The crucial role of selectivity in cancer chemotherapy may be found in pharmacological targeting of natural molecules avoiding cytotoxicity against normal cell. Evidences from in vitro and in vivo studies have shown that polyphenol-rich food consumption is associated with a less risk for cardiovascular disease and cancers. The phenolic compounds are reported to have numerous biological effects including antioxidant, antiproliferation activity. Recently, studies have been focused on the naturally occurring phenol compounds that are able to decrease the generation of ROS in biological system (22).

Lactic acid fermentation is a traditional food fermentation method that produces useful substances by lactic acid bacteria and provides health benefits such as anti-bacterial, anticancer, skin diseases, anti-inflammation and anti-virus (1). Lactic acid bacteria used for fermentation with plant-based foods are mostly used as methods for preserva-

tion, enhanced nutritional quality and health benefits, production of bioactive compounds. The studies on the activities of fermented fruits using lactic acid bacteria have been actively conducted. For example, lactic fermentation has been applied to enhance the antioxidant activity in tropical fruit juices and teas (2). Ravish B et al. (3) reported that the fermentation of raw guava fruit extract by lactic acid bacteria increased in antioxidant activity and content of total phenolics. A study on the antioxidant activity of fermented hawthorn (4) and on phenolic compound biotransformation of mulberry fermentation (5) were also reported. In addition, the previous studies have shown that *Lactobacillus plantarum* isolated from kimchi contain tannase activities that degrade tannin. These tannase activities have been reported to cause hydrolysis of phenol and flavonoid compounds and destruction of plant cell walls, leading to the synthesis of various bioactive compounds related to antioxidant and anticancer (14).

Blueberries belongs to the Ericaceous Vaccinium group of deciduous shrub plants and are widely cultivated in many countries around the world. Blueberries are three primary blueberry species, including cultivated rabbiteye (V. ashei), highbush (V. corymbosum), and lowbush (V. angustifolium) blueberries (6). It is mainly distributed in North America, and as interest in functional foods increases, production and demand for blueberries in South Korea are also increasing (7). Blueberries are a rich source of dietary bioactivities compounds including phenolic compounds, flavonol, flavanol, tannin, vitamin C and especially anthocyanin (8). Various of pharmacological activities, especially antioxidant (9), anticancer (10), anti-inflammation (11), anti-obesity (12), anti-

diabetic activities (13) of bluberry have been reported. However, only studies related to antioxidant (15, 16), anti-diabetes (17) and volatile compounds change (18) related to blueberry fermentation by lactic acid bacteria were performed. Therefore, this study was undertaken to examine the effects of blueberry fermentation on both antioxidant and antiproliferative activity using *Lactobacillus plantarum* containing the enhanced tannase activities. We aimed to confirm the changes of phenolic compounds, antioxidants and antiproliferative activities of blueberries fermentation using *Lactobacillus plantarum* CK10, thus providing basic data for development of functional food made from the fermented blueberry.

2. Materials and Methods

2-1. Reagents

The Folin-Ciocalteu phenol reagent, gallic acid, rutin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2'-azobis(2-methylpropionamid) dihydrochloride (AAPH), α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (4-POBN), pyrogallol, propidium iodide (PI), protocatechuic acid, catechol, chlorogenic acid, ferulic acid, delphinidin-3-O-glucoside, RNase A and β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), 100x penicillin/streptomycin solution were purchased from Gibco (Grand Island, NY, USA). Hoechst 33342 dye, dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Amresco Inc. (Solon, OH, USA). Annexin V-FITC Apoptosis Detection Kit-1 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). PARP, c-PARP caspase-7 and -9, c-caspase-9 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). A BCA protein assay kit was purchased from Pierce (Rockford, IL, USA), and polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). For GC-MS analysis, high-performance liquid chromatography (HPLC)

grade solvents were purchased from Merck Inc. (Darmstadt, Germany). All standard chemicals and reagents were of analytical grade.

2-2. Plant materials

Blueberry fruits from rabbiteye blueberry (*Vaccinium ashei* species) were purchased from local blueberry orchards in Jocheon-eup, Jeju Island on September of 2016. Fruits with no physical damage and uniform size and color were used for the following experiments.

2-3. Fermentation processes and Extraction

2-3-1. Bacterial strains and culture conditions

Lactobacillus plantraum CK10 isolated from an *Oisobaki* (cucumber kimchi) was used in the present study. *L. plantraum* CK10 showed high tannase activity (11). Strains were propagated into de Man, Rogosa and Sharpe (MRS) broth (BD Difco, Sparks, MD, USA). Strains were cultured at 30℃, 100 rpm condition for 24 h.

2-3-2. Blueberry-based media and growth of *lactobacillus plantraum* CK10

The production of blueberry-based media was carried out by a modified method of Daniela et al. (23). It was grown on blueberry-based media containing (%, w/v): 5% blueberry powder, 2% peptone water (BD Difco, Sparks, MD, USA) in distilled water

(D.W). This media was bring to pH 6.0 ±0.5 with 1N NaOH and sterilized at 121 °C for 15 min. Before the inoculum, *L. plantarum* CK10 was inoculated (1.0%, v/v) and sub-cultured twice in MRS at 30 ℃ for 24h. Stains were inoculated in the media at the final cell density of 7.00 ± 0.50 Log CFU/mL. Fermentation was carried out for 7 days at 30 ℃, 100 rpm condition.

2-3-3. Extraction

Briefly, fermentation products were extracted with 80% Ethanol at room temperature with sonication for 45 min. The extracts were filtered using filter paper and concentrated with a vacuum rotary evaporator under reduced pressure at 35 ℃. Each extract was lyophilized to obtain the powder and stored at -20 ℃ before analysis. Non-fermented blueberry extract (NFBE) was processed in an identical manner but was not fermented with *L. plantarum* CK10.

2-4. Viability and chemical characterization of blueberry fermented by *L. plantarum* CK10

The colony forming unit (CFU) was determined in the fermentation products by plating onto MRS agar and incubation at 37 ℃ for 2 days. Prior to plating, serial dilutions were made by homogenizing 100 μL of each sample with 900 μL phosphate buffer solution (PBS). The pH was determined with an Orion 3star bench top pH meter

(Thermo Scientific Inc., Waltham, Mass., USA). The total titratable acidity (TTA) was titrated up to pH 8.2 with a 0.1N NaOH. The total soluble solid (TSS) was measured using a brix refractometer (PAL-1, Atago, Japan).

2-5. Determination of the total polyphenol and flavonoid contents

The total polyphenol content was determined by a slightly modified method of Cheung et al. (24). A 125 μL aliquot of the extracts were mixed with 0.5 mL of Folin–Ciocalteu's phenol reagent. After 5 min, 1 mL of 10% Na_2CO_3 (w/v) was added to the mixture. The reaction was performed in dark conditions for 30 min, after which its absorbance was recorded at 700 nm using a microplate reader (Sunrise, Tecan, Salzburg, Austria). Results were expressed as mg gallic acid equivalent (GAE)/g dry weight on the basis of the dried sample. Total flavonoid content was measured by method of Zhishen et al. (25). The absorbance was measured at 510 nm using a microplate reader (Sunrise, Tecan, Salzburg, Austria). Results were expressed as mg rutin equivalent (RE)/g dry weight on the basis of the dried sample.

2-6. Antioxidant activity

2-6-1. Radical scavenging activity by DPPH assay

DPPH radical scavenging activity was measured based on the previously reported methods (26, 27). The DPPH radical solution was prepared by dissolving DPPH in ethanol at 200 μM, and 40 μL of each extracts (2mg/mL in DMSO) was dispensed into a 96-well plate. After adding 160 μL of 200 μM DPPH radical solution, the reaction was carried out at 37 °C for 30 minutes. At the end of the reaction, the absorbance was measured at 517 nm using a spectrophotometer (Sunrise, Tecan, Salzburg, Austria). Catechin was used as a positive control.

2-6-2. Radical scavenging activity by ABTS assay

ABTS radical scavenging activity was performed according to Re R. et al. (28). The ABTS radical cation radical solution was generated by adding 7 mM ABTS to a 2.45 mM potassium persulphate solution and allowing the mixture to stand in dark conditions at room temperature for 16 h before use. To obtain an absorbance of 0.700 ± 0.005 at 734 nm measured with a UV 1800 spectrophotometer (Shimadzu, Kyoto, Japan), the stock solution was diluted with distilled water. Further, 100 μL of the extracts (2 mg/mL in DMSO) was added to 900 μL of this diluted stock solution, and the absorbance was determined after 2 min of initial mixing. The percentage of scavenging activity was calculated as the percentage reduction in absorbance.

2-6-3. Radical scavenging activity by Alkyl assay

Alkyl radical solution was generated by reaction mixtures containing 40 mM AAPH and 40 mM 4-POBN (29). This solution and extracts (500 μg/mL in DMSO) mixture

were incubated at 37 ℃ in a water bath for 30 min, and then transferred to 50 μL Teflon capillary tubes. The radical scavenging activity was measured using a JES-FA200 ESR spectrometer (JEOL, Tokyo, Japan). The measurement conditions were as follows: magnetic field, 336.000 mT; power, 7 mW; sweep time, 30 s; sweep width, 10 mT; frequency, 9.43 GHz; modulation width, 0.2 mT; time constant, 0.03 s. The radical scavenging activities were calculated according to the following formula:

$$\frac{Control - Sample}{Control} \times 100(\%)$$

Signal intensity was compared on the basis of the ratio against the magnetic marker (standard of ESR, Mn2+ marker) and was represented by relative height ratio.

2-6-4. Superoxide dismutases (SOD)-like activity

SOD-like activity was measured by Marklund's method (30). A 50 μL of extracts (4 mg/mL in DMSO) were added 50 μL of 7.2 mM pyrogallol, 50 μL of Tris-HCl buffer (50 mM tris[hydroxymethyl] aminomethane + 10 mM EDTA, pH 8.5) and incubate at 25 ℃ for 45 min. At the end of the reaction, the absorbance was measured at 420 nm using a spectrophotometer (Sunrise, Tecan, Salzburg, Austria).

2-7. Antiproliferative activity

2-7-1. Cell culture

The human cervical carcinoma HeLa cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Human Dermal fibroblast were obtained from donors who underwent routine circumcision at Jeju National University Hospital, Korea. The cells were cultured in Dulbecco's Modified Eagle Medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics and were maintained in a humidified incubator at 37 ℃ in a 5% CO_2 atmosphere.

2-7-2. Determination of the effect on in vitro cell viability

2-7-2-1. MTT assay

The effect of fermented blueberries on the viability was determined by MTT assay, according to a previously described methods (31). Briefly, cells (2×10^4 cells/mL) were seeded in 96-well culture plates. After 24 h incubation, the cells were treated with various concentrations of the different extracts. Then, 20 μL of MTT solution (5 mg/mL) was added to each well, and the cells were incubated at 37 ℃ for 2h. The supernatant was removed and dissolved in 150 μL DMSO. Absorbance was detected in a microplate reader at 570 nm (Tecan, Salzburg, Austria). The percentage of cell viability was calculated as the percentage reduction in absorbance.

2-7-2-2. Colony formation assay

HeLa cells were seeded in 6-well plates at 1,000 cells/well and exposed to the extracts for 7 days. After treatment, cells were fixed with 4% paraformaldehyde and stained

with crystal violet for 15 min.

2-7-3. Cell morphology analysis

HeLa cells were placed in 60 mm dish at 3×10^4 cells/mL and treated with 2 mg/mL concentration of the extracts. After 24h, the treated cells were stained with 10 μM Hoechst 33342 for 10 min at 37 °C. The stained cells were then observed under a fluorescence microscope (Olympus, UK).

2-7-4. Flow cytometry

For sub-diploid population detection, cells were detached from plates by the addition of trypsin-EDTA, washed in phosphate-buffered saline (PBS), fixed in 70% ethanol, treated with RNase A (25 ng/mL), and stained with propidium iodide (PI) (40 μg/mL). The annexin V-FITC apoptosis detection kit I was used according to the manufacturer's protocol to detect phosphatidylserine translocation from the inner to the outer plasma membrane. For each assay, cells were washed with PBS, diluted in annexin V binding buffer containing annexin V and PI, and incubated for 15 min at room temperature. Data from 10,000 cells/sample were analyzed with Cell Quest software (Becton Dickson, USA). Each experiment was repeated at least 3 times.

2-7-5. Western blot analysis

Cell lysates were prepared by incubating cells in 200 μL of lysis buffer (100 mM Tris-

HCl, pH 8, 250 mM NaCl, 0.5% Nonidet P-40, 1× protease inhibitor cocktail), disrupted by sonication, and extracted at 4°C for 25 min. The protein concentration was determined using BCA^{TM} protein assays (Pierce, Rockford, IL, USA). Aliquots of the lysates were separated using 12–15% SDS-PAGE and transferred to PVDF membranes using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl pH 8.8, and 20% methanol (v/v). After blocking with 5% non-fat dried milk, the membrane was incubated for 24 h with primary antibodies followed by 40 min with secondary antibodies in Tris-buffered saline (TBS) containing 0.5% Tween-20. Most primary antibodies were used in 1:1,000 dilutions except for β-actin (1:4,000 dilution), and the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were used in 1:5,000 dilutions. Protein bands were detected using the BS ECL-Plus Kit (Biosesang, Gyeonggi-do, Korea).

2-8. Determination of volatile compounds by GC-MS

Identification of volatile compounds were carried out by Shimadzu gas chromatography mass spectrometry (GC-MS; Model QP-2010, Shimadzu Co., Kyoto, Japan) in Electron Impact (EI) mode. The ionization voltage was 70 eV, and injector and interface temperatures were 250°C and 290°C, respectively. The capillary column was an Rtx-5MS (30 m length, 0.25 mm i.d., and 0.25 μM, film thickness). The oven temperature, programmed at 60°C (isothermal for 3 min), was ramped to 300°C at 5°C/min

(isothermal for 3 min) and 310°C at 10°C/min (isothermal for 5 min). Helium, the carrier gas, was used at a flow rate of 1 mL/min with 57.4 kPa pressure and an injector volume of 1 μL using splitless mode. Mass range was from m/z 40-800 amu. The MS spectral data were compared within WILEY9 libraries.

2-9. Determination of lactic acid and phenolic compounds by HPLC

For measurement of lactic acid were quantified using an Ultra-fast liquid chromatography-diode array detector (UFLC-DAD, Shimadzu Co., Japan) equipped with a shim-pack GIS column (250x4.6 mm, 5μm ODS, shimadzu Co., Kyoto, Japan), a quaternary pump, an auto sampler. Separation was performed using 10 mM potassium phosphate in D.W (adjusted to pH 2.4) (A) and acetonitrile (B) as eluents at a flow rate of 0.8 mL/min with the following gradient: 0-10 min, 0% B; 10-15 min, 5% B; 15-30 min, 10% B; 30-50 min, 15% B; 50-55 min, 15% B; 55-60 min, 5% B; 60-65 min, 0% B. The injection volume was 20 μL and the temperature of column was maintained at 30 ℃. The detector was set to 205 nm.

To determine the phenolic compounds, the operational parameters of the UFLC-DAD were set as described above. Gradient elution was performed using 0.1% formic acid in D.W (A) and acetonitrile (B) as eluents at a flow rate of 0.8 mL/min with the following gradient: 0-5 min, 0% B; 5-10 min, 3% B; 10-15 min, 5% B; 15-20 min, 10% B; 20-25 min, 20% B; 25-30 min, 30% B; 30-35 min, 30% B; 35-40 min, 20% B; 40-

45 min, 10% B; 45-50 min, 0% B; 50-55 min, 0% B. The injection volume was 20 μL and the oven temperature was set at 35 ℃. The detector was set to 254 nm.

2-10. Determination of anthocyanin content by HPLC

To determine the anthocyanin content, anthocyanins were extracted from blueberry extracts according to Qixing et al. (32). The operational parameters of the UFLC-DAD were set as described above. Gradient elution was performed using 10% formic acid in D.W (A) and methanol (B) as eluents at a flow rate of 1 mL/min with the following gradient: 0-10 min, 7% B; 10-15 min, 15% B; 15-20 min, 25% B; 20-30 min, 7% B. The injection volume was 20 μL and the oven temperature was set at 30 ℃. The detector was set to 520 nm.

2-11. Statistical analysis

All experiments were repeated three times and the mean and standard deviation were obtained using (Statistical package for social sciences, SPSS Inc, Chicago, IL, USA) software (version 18.0). The significance of differences between groups was determined through one-way analysis of variance (ANOVA).

3. Results

3-1. Growth of *L.plantarum* CK10 on blueberry-based media

In order to evaluate the growth of *L.plantarum* CK10 in blueberry-based media, growth behavior and metabolism of *L.plantarum* CK10 was monitored (Table 1). The cell viability of *L.plantarum* CK10 increased after 2 days (initial cell viability 7.38 Log CFU/mL) reaching values 9.60 Log CFU/mL. After 7 days of fermentation, cell viability decreased from 9.60 to 7.30 Log CFU/mL. The changes in pH, TTA, TSS and lactic acid content during fermentation, the results of the microbial growth, were determined. The initial values were 6.00, 0.30%, 6.00 Brix %, 10.43 mg/g weight for pH, TTA, TSS and Lactic acid content, respectively. During fermentation time, the pH values gradually decreased, passing from 6.00 to 3.48 after 7 days. The TTA values slightly increased during the 7 days of fermentation, passing from 0.30 to 1.34 %. In contrast, the TSS values decreased during the first day of fermentation, passing from 6.00 to 5.50 Brix %, and remained until the end of the fermentation time. Conversely, the lactic acid content significantly increased in blueberry-based media, passing from 10.43 to 106.15 mg/g weight.

Table 1. Determination of viability, pH value and chemical characteristics during fermentation time with *Lactobacillus plantarum* CK10

	Fermentation time (Days)				
	0	**1**	**2**	**3**	**7**
Viability (Log CFU/mL)	7.38	9.00	9.60	8.80	7.30
pH value	6.00	3.78	3.60	3.53	3.48
Total titratable acidity (%)	0.30	0.69	1.01	1.14	1.34
Total soluble solid (Brix %)	6.00	5.50	5.30	5.30	5.40
Lactic acid (mg/g weight)	10.43	47.28	70.32	85.54	106.15

3-2. Determination of total polyphenol and flavonoid contents

Phenolic compounds could be the major determinants of the antioxidant activity of various plants (33). Therefore, the contents of total polyphenol and flavonoid were measured and expressed as mg gallic acid equivalent (GAE)/g and mg rutin equivalent (RE)/g of fermented blueberry extracts (FBE), respectively (Table 2). During fermentation time, the contents of polyphenol and flavonoid gradually increased, passing from 1.57±0.05 to 2.06±0.04 mg GAE/g and from 0.64±0.06 mg to 1.11±0.09 mg RE/g, respectively.

3-3. Antioxidant activities of FBE

The antioxidant activities of fermented blueberry extracts (FBE) were determined based on the scavenging activities towards DPPH, ABTS, and Alkyl radical scavenging activities, SOD-like activity (Table 2). The results revealed that blueberry extracts gradually were increased antioxidant activities during fermentation time. The blueberry extract fermented for 7 days had the highest DPPH, ABTS, Alkyl and SOD values, which were 52.85 ± 3.20 %, 61.51 ± 0.91 %, 59.36 ± 5.72 %, 53.72 ± 6.42 %, respectively. The results revealed that as the values of TPC and TFC increased, the antioxidant activities also improved.

Table 2. Total polyphenol and flavonoid contents, Antioxidant activities during fermentation time

Fermentation time (Days)	TPC (mg GAE/g)	TFC (mg RE/g)	Antioxidant activities			
			DPPH (%)	ABTS (%)	Alkyl (%)	SOD (%)
0	1.50 ± 0.01^{a}	0.61 ± 0.01^{a}	34.11 ± 2.43^{a}	52.74 ± 0.72^{a}	45.89 ± 5.32^{a}	-1.67 ± 4.00^{a}
1	1.56 ± 0.01^{b}	0.72 ± 0.01^{b}	37.71 ± 3.11^{b}	49.02 ± 0.09^{b}	45.88 ± 8.61^{a}	22.47 ± 4.62^{b}
2	1.79 ± 0.01^{c}	0.88 ± 0.01^{c}	44.09 ± 3.71^{c}	53.59 ± 1.05^{a}	48.71 ± 3.82^{a}	36.72 ± 2.70^{c}
3	1.85 ± 0.00^{d}	1.01 ± 0.02^{d}	50.22 ± 2.98^{d}	57.47 ± 0.91^{c}	54.59 ± 7.36^{ab}	44.65 ± 4.27^{d}
7	2.02 ± 0.01^{e}	1.17 ± 0.01^{e}	52.85 ± 3.20^{e}	61.51 ± 0.91^{d}	59.36 ± 5.72^{b}	53.72 ± 6.42^{e}

Letters represent significant differences among extracts by Least Significant Difference (LSD) test $p<0.05$. TPC: Total phenol content; GAE: Gallic acid equivalents; TFC: Total flavonoid content; RE: Rutin equivalents; DPPH: DPPH radical scavenging activity; ABTS: ABTS radical scavenging activity; Alkyl: Alkyl radical scavenging activity; SOD: SOD-like activity.

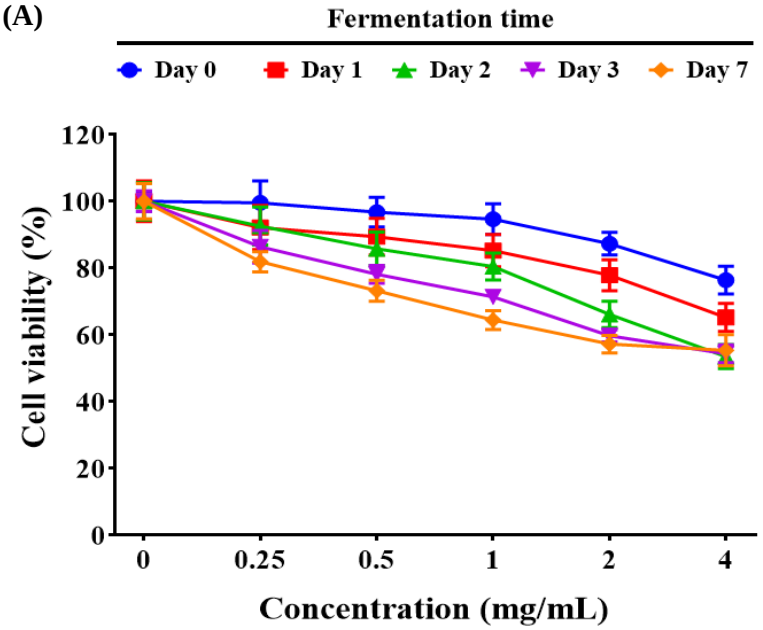
3-4. Antiproliferative activities of FBE

3-4-1. Effects of FBE on cytotoxicity and colony formation ability in HeLa cells

To investigate the effects of FBE on human cervical carcinoma HeLa cells, cells were treated with various concentrations of FBE for 24h and percentage HeLa cell death was determined using MTT assays (Figure 1 (A), (B)). The results exhibited that FBW reduced the viability of HeLa cells in fermentation time-, dose-dependent manner but did not reduce normal cells.

To determine the effects of FBE on HeLa cell regrowth, the colony formation assay was conducted (Figure 1 (C)). The results revealed that FBE decreased colony forming ability in fermentation time-, dose-dependent manner. FBE exhibited cytotoxic and antiproliferative effects against the HeLa cells according to fermentation time.

(A)



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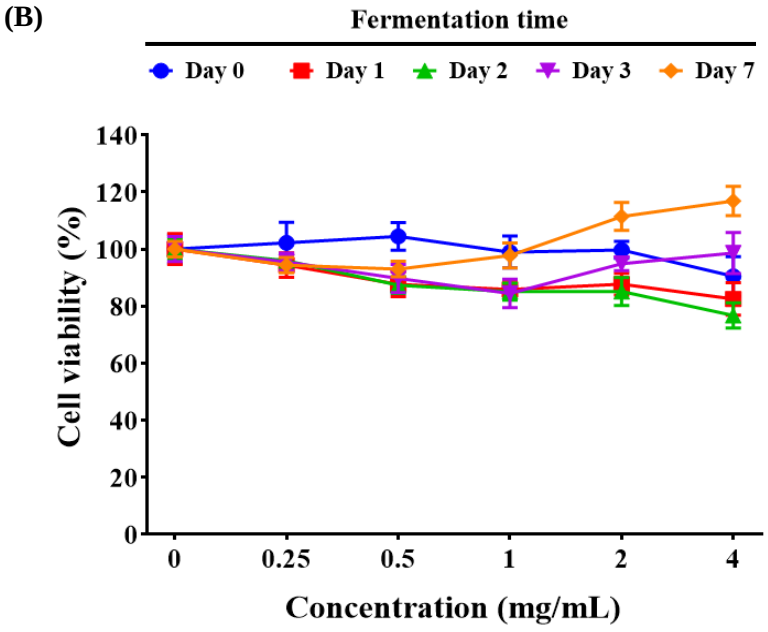


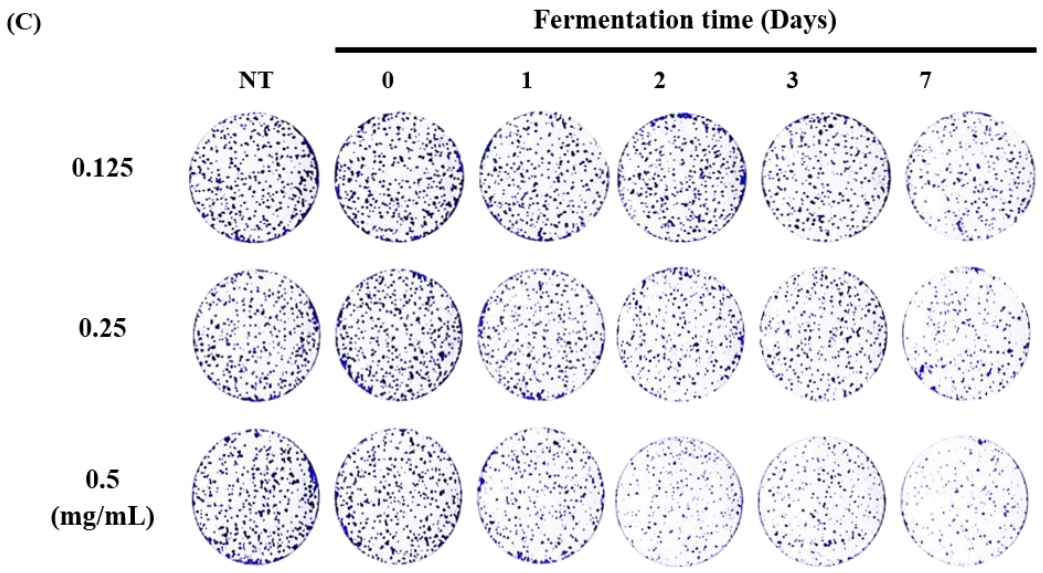


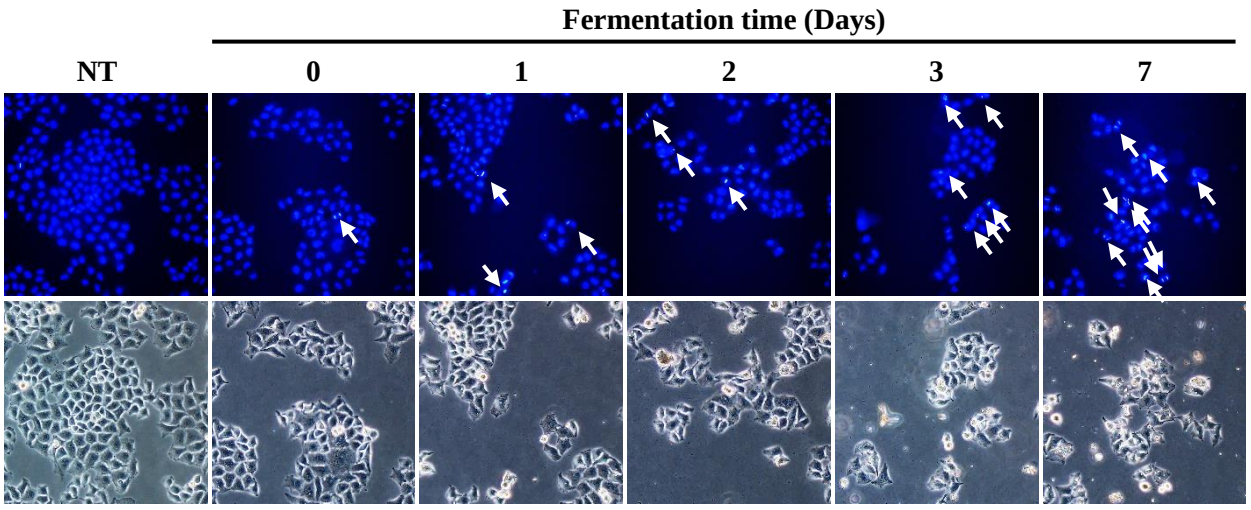
Figure 1. Effects of FBE on cytotoxicity and colony formation ability in HeLa cells

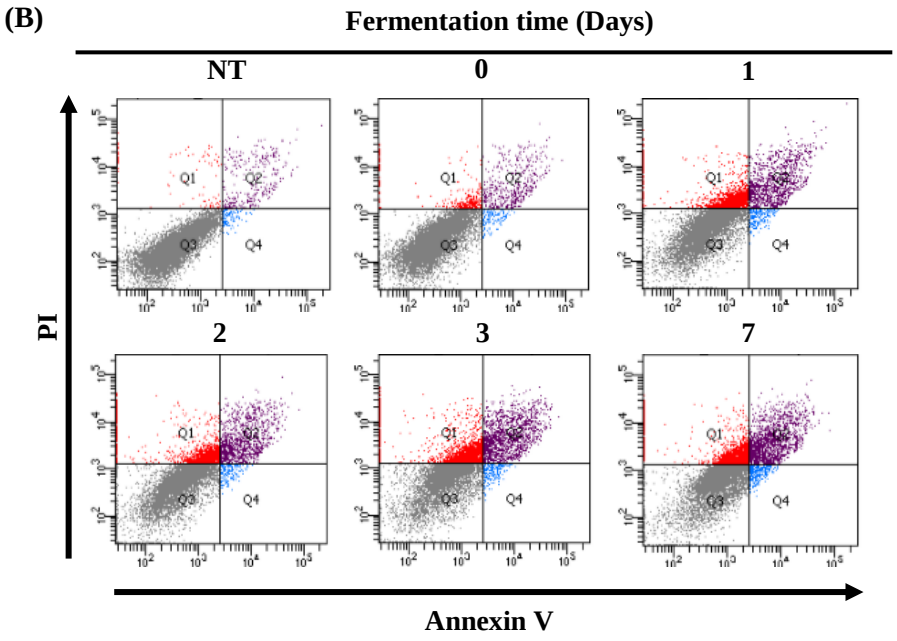
(A) Fermented blueberries induced cytotoxicity in HeLa cells. Cell were treated with the indicated concentrations of fermented blueberries for 24h, and MTT assays were performed. (B) Normal human dermal fibroblast cells were treated with the indicated concentrations of fermented blueberries for 24h. (C) HeLa cells were treated with various concentrations of fermented blueberries for 7 days, and the cell viability was determined by crystal violet staining.

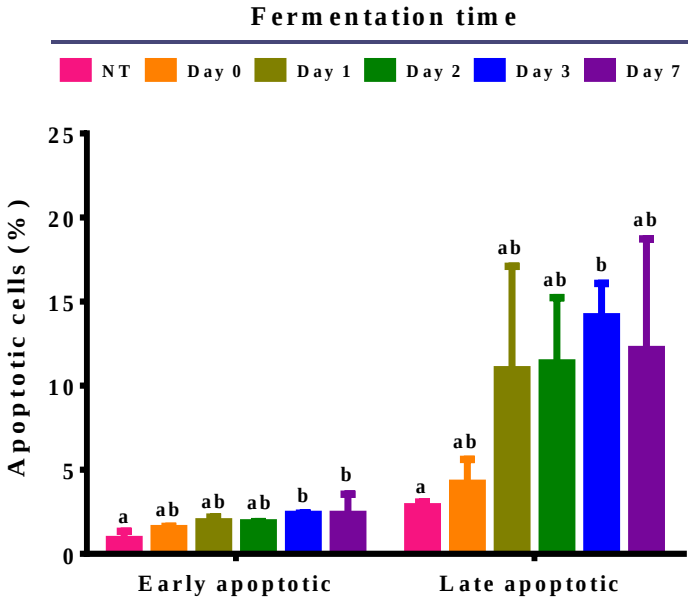
3-4-2. FBE induced apoptosis in HeLa cells

To investigate the mechanism of FBW induced in HeLa cells, HeLa cells were treated with the FBE, stained with Hoechst 33342, and then observed under fluorescence microscopy (Figure 2 (A)). After treatment with the FBE, increase in apoptotic cells was observed in the FBE, whereas NFBE showed few apoptotic cells. Furthermore, cell apoptosis was determined by Annexin V/PI staining (Figure 2 (B)). The result showed that compared with NFBE (5.70 %), the percentage of total apoptotic HeLa cells after FBE treatment was as follows: 12.85 % (Day 1), 13.20 % (Day 2), 16.45 % (Day 3), 14.50 % (Day 7). Consistent with above results, the percentage of sub-G1 phase (apoptotic cells) were increased after cells were treated with FBE up to 18.06 and 25.18 % respectively compared with 10.02 % in the NFBE (Figure 2 (C)). These above results revealed that FBE was effective in apoptosis induction of HeLa cells compared with NFBE.

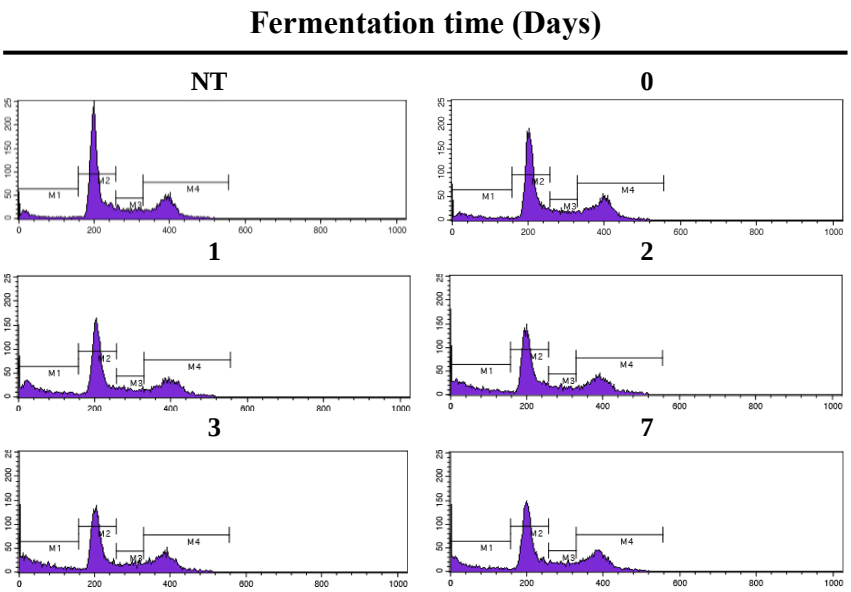
(A)







(C)



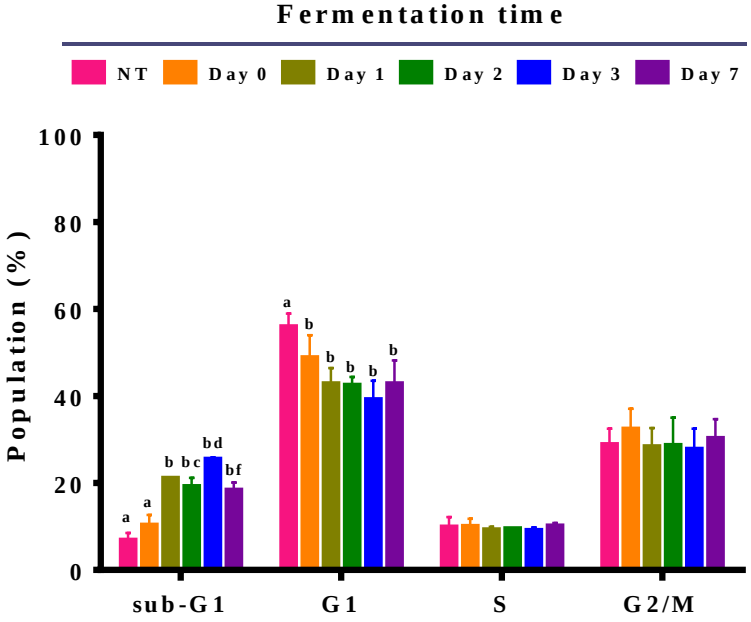


Figure 2. FBE induced apoptosis in HeLa cells

Cells were treated with the 2 mg/mL of fermented blueberries, D.W (NT) for 24h. (A) Apoptotic bodies (arrows) were observed and quantitated by fluorescence microscopy in cells stained with Hoechst 33342 dye. (B) The proportion of apoptotic cells was determined by double-staining with Annexin V/FITC and PI. The flow cytometry profile presents Annexin V-FITC (x-axis) and PI staining (y-axis). (C) Flow cytometric analysis was performed for cell-cycle distribution. The DNA content was evaluated with propidium iodide (PI) staining and fluorescence measured and analyzed.

3-4-3. Effect of FBE on the expression of apoptosis related protein

Apoptosis is form of the cell death characterized by morphological change and activation of cystein-asparate protease (caspase) and pro-apoptotic protein. In intrinsic pathway, caspase-9 was cleavage by Apaf-1 and cytochrome C then finally executioner caspase-3 and -7 were activated (34). PARP is a zinc-finger DNA-binding protein which catalyzes the synthesis of poly(ADP-ribose) from its substrate β-NAD+ and is implicated in the maintenance of genomic stability and DNA damage-triggered signaling cascade. PARP can be selectively cleaved by caspase during apoptosis and become incapable of responding to DNA damage. Since PARP is one of the potential target molecules of effector caspases, the PARP cleavage has been regarded as an evidence of caspase activation and has been widely used as a hallmark of cell apoptosis (35). To further investigate FBW affected the apoptosis in HeLa cells, we determined activation of caspase -7, -9 and PARP cleavage by western blot analysis (Figure 3). The results indicated the fermentation time-dependent caspase-7, -9 activation and cleavage of PARP after FBE treatment. Overall, the results suggested that caspase-mediated apoptosis was induced in HeLa cells by FBE compared with NFBE.

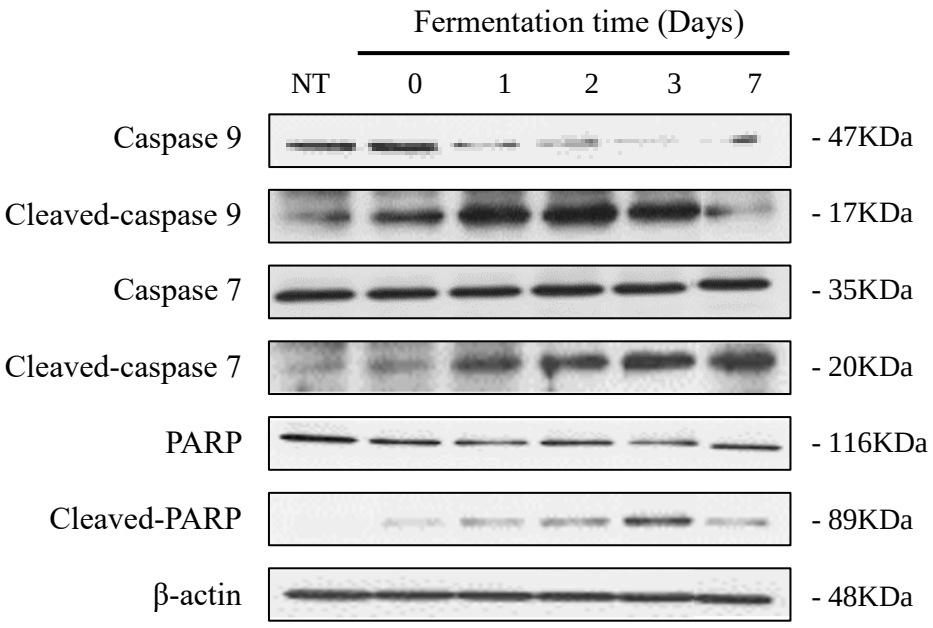


Figure 3. Effect of FBE on the expression of apoptosis related protein

Lysates were prepared from cell treated with the 2 mg/mL of fermented blueberries, D.W (NT) for 24h, and then western blotting was performed using caspase-7, caspase-9, and PARP antibodies.

3-5. Changes in volatile compounds during fermentation time

These data suggest that FBE-derived phytochemicals play a role in inhibiting HeLa cell proliferation. Thus, the FBE was analyzed using GC-MS analysis. The GC-MS analysis revealed total 30 compounds, including lactic acid, alpha,beta-crotonolactone, 1,2-cyclopentanedione, phenol (Table 3). The main constituent of FBE was identified as lactic acid, 1, 2-Benzenediol compared with NFBE. Interestingly, FBE contained 1, 2-Benzenediol, also known as pyrocatechol, was reported to found in fruits and vegetable such as apples and onions. 1, 2-Benzenediol was reported to inhibit cancer growth in human lung cancer cells. Also, some phytochemicals containing the catechol moiety, such as quercetin, luteolin showed anticancer activity (36). It is possible that health-beneficial effects are expressed by the constituents of FBE, in which case the compounds, either individually or synergistically, would be responsible for the antioxidant and antiproliferative activities.

Table 3. Changes in the volatile compounds during fermentation time

No.	RT [a]	Compounds [b]	Peak area % [c]				
			0	1	2	3	7
1	8.575	Lactic acid	-	29.93	58.22	75.43	77.52
2	8.732	.ALPHA.,.BETA.-CROTONOLACTONE	5.00	1.37	-	-	-
3	8.875	1,2-Cyclopentanedione	9.87	4.65	1.54	-	-
4	9.913	Phenol	-	-	0.68	0.40	0.27
5	10.261	Formic acid, 2-propenyl ester	15.58	6.87	3.28	1.40	0.77
6	10.728	2,5-Furandione, dihydro-3-methylene-	-	0.30	0.16	0.10	0.09
7	10.901	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	1.47	0.49	0.16	0.08	0.08
8	11.538	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	3.30	1.37	0.43	0.22	0.14
9	12.293	Phenol, 2-methoxy-	-	0.43	0.20	0.12	0.09
10	12.4	Propanoic acid, 2,2-dimethyl-	-	1.52	0.47	0.12	0.11
11	13.713	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	5.94	5.71	4.12	2.68	2.22
12	14.221	Benzoic acid	-	0.69	0.49	0.34	0.35
13	14.825	4H-Pyran-4-one, 3,5-dihydroxy-2-methyl-	-	-	0.37	0.21	0.10
14	15.065	1,2-Benzenediol	-	14.21	9.56	6.03	6.38
15	19.17	Phenol, 2,6-dimethoxy-	0.36	1.05	0.51	0.31	0.31
16	20.629	Junipene	0.39	-	-	-	-
17	26.239	Quinic acid	31.87	13.00	9.30	5.91	5.21
18	29.44	1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	8.66	5.87	4.12	2.68	2.22
19	32.414	Xycaine	2.38	1.80	1.16	0.71	0.47
20	32.722	Methyl palmitate	0.46	0.19	0.07	0.03	0.03
21	32.949	1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	-	0.33	0.27	0.17	0.12
22	33.451	Palmitic acid	-	0.17	0.10	0.05	0.08

23	36.121	Methyl linoleate	1.03	0.37	0.08	0.05	0.03
24	36.255	Methyl linolenate	0.74	0.26	0.05	0.04	0.02
25	37.665	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	2.56	0.74	1.00	0.04	0.03
26	43.344	Methyl palmitoleate	1.18	-	-	-	-
27	48.355	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapen-taene	1.01	0.34	0.18	0.11	0.10
28	56.1	Stigmast-5-en-3-ol, (3.beta.)-	2.52	3.14	1.39	1.00	1.02
29	56.833	METHYL COMMATE A	1.35	1.27	0.52	0.44	0.56
30	57.666	METHYL COMMATE D	4.33	3.93	1.57	1.33	1.68

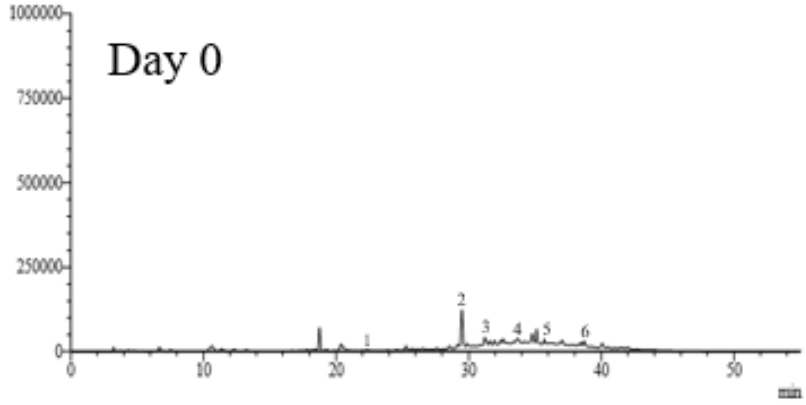
[a)] Retention time

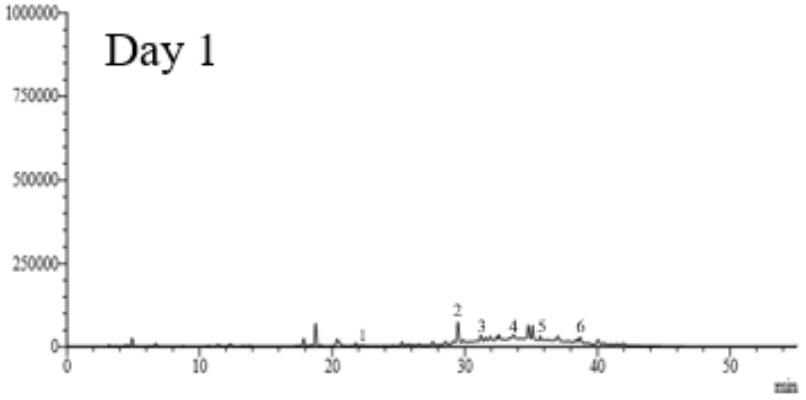
[b)] Compounds tentatively identified based on parent molecular ions, retention times, retention indices and elution order, and fragmented spectra compared with the literature

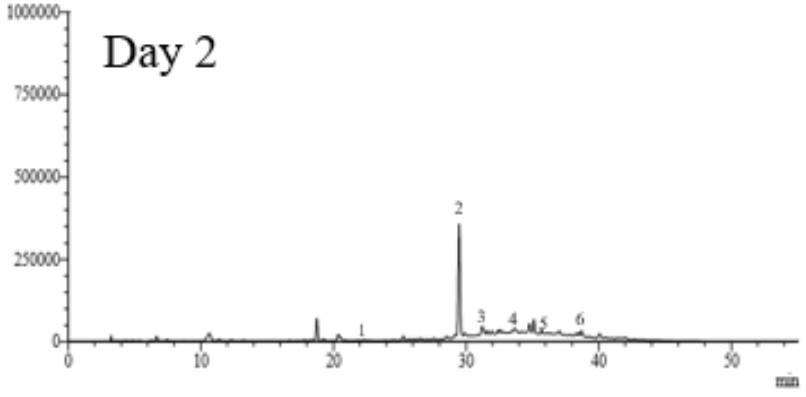
[c)] Peak area percentage (peak area relative to the total peak area %)

3-6. Changes in phenolic compounds during fermentation time

The HPLC allowed the separation, identification and quantitation of five phenolic acids (gallic acid, protocatechuic acid, catechol, chlorogenic acid, ferulic acid), one flavonoid (rutin) and one anthocyanin (delphinidin 3-glucoside) in FBE (Table 4). All the identified compounds in this investigation were previously reported in blueberries (8, 10). Chromatograms of phenolic compounds of FBE in Figure 4. In comparison with NFBE, delphinidin 3-glucoside, gallic acid did not show any difference in FBE. Whereas, protocatechuic acid was significantly increased, passing from 314 to 2139 μg/g weight after 7 days. Also, catechol, chlorogenic acid, ferulic acid, and rutin were increased by fermentation.







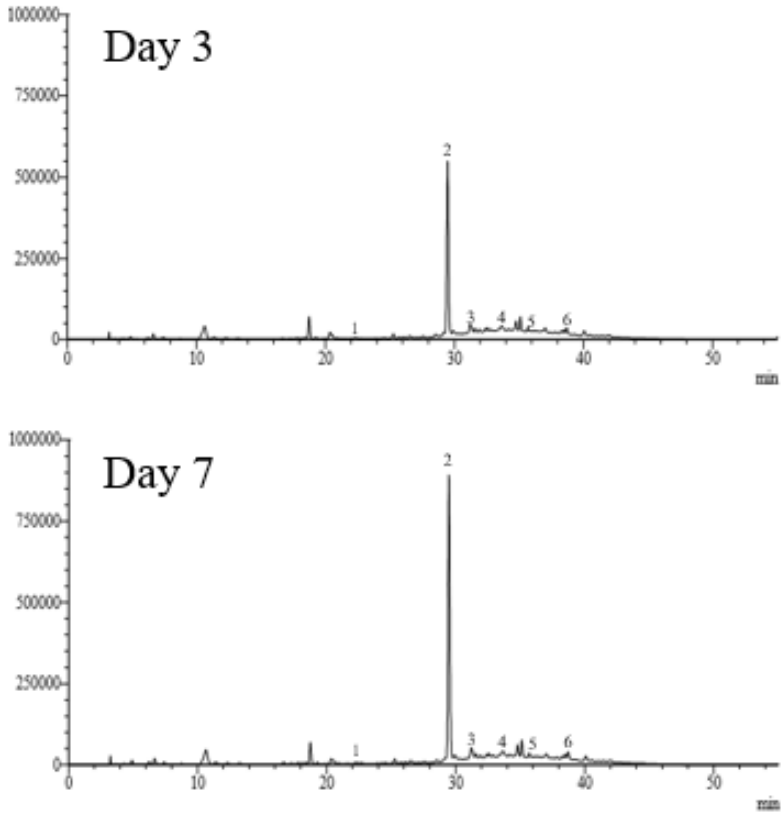


Figure 4. HPLC chromatograms of FBE for 7 days

chromatograms at 254 nm. peak 1: gallic acid; peak 2: protocatechuic acid; peak 3: catechol; peak 4: chlorogenic acid; peak 5: rutin, peak 6: ferulic acid.

Table 4. Contents of delphinidin 3-glucoside, phenolic compounds (μg/g weight) during fermentation time

Fermentation time (Days)	Anthocyanin	Phenolic compounds					
	Delphinidin 3-glucoside	Gallic acid	Protocatechuic acid	Catechol	Chlorogenic acid	Ferulic aicd	Rutin
0	91.60	43.16	313.88	537.94	88.95	174.49	291.95
1	88.72	31.10	435.54	850.93	121.43	150.12	335.95
2	96.36	30.22	1028.23	1044.81	124.47	171.15	340.81
3	101.14	28.74	1214.82	877.08	138.03	137.41	359.00
7	66.76	36.14	2318.61	1236.65	156.23	226.89	412.00

4. Discussion

Many reports have indicated that the consumption of fruits and vegetables was correlated with the decreased risks of some disease. The plant kingdom is a valuable source of bioactive compounds and phytochemicals. Numerous reports have detailed their potential positive benefits for human health (8). The berries, an important fruits group that is mainly preferred for their specific sweet-sour taste, aroma and colorful fruits are mainly investigated not only for their nutritional properties but also for their bioactive constituents (37). The berries, including blueberry, cranberry, raspberry, and strawberry are good sources of flavonols while the predominating group of flavonoids, especially in red berries, is anthocyanins (38). Blueberries consist of a large array of bioactive compounds that include polyphenols, phenolic compounds, and stilbene derivatives. Antioxidant compounds in blueberry can be known to reduce the risk of coronary, cardiovascular and neurodegenerative diseases (39). *L. plantarum CK10* containing tannase is an enzyme that catalyzes the breakdown of ester bonds in gallotannins such as tannic acid. By this tannase, tannic acid is decomposed into gallic acid and protocatechuic acid (40). In the present study, we sought to determine the polyphenol and flavonoid contents, antioxidant and antiproliferative activities in fermented blueberry extracts. This study was the first to report the antiproliferative activity of fermented blueberries in HeLa cells. As a first step, we made the blueberry-based media containing peptone water and fermented using *L. plantraum* CK10. The fermented

blueberries were extracted and freeze-dried. The total polyphenol and flavonoid contents, free radical scavenging activities and SOD-like activity of fermented blueberry extracts increased higher than non-fermented blueberry extracts. Also, fermented blueberry extracts inhibited the growth and induced apoptosis in HeLa cells. In our study, we found that fermented blueberry extracts decreased full length caspase-9, caspase-7 and PARP. The changes in volatile compounds during fermentation time were confirmed by GC-MS analysis, lactic acid, catechol were increased in fermented blueberry extracts. And changes in phenolic compounds during fermentation time were identified by HPLC analysis, the increase of protocatechuic acid, catechol, chlorogenic acid, ferulic acid, and rutin was confirmed in fermented blueberry extracts. Our results showed that anthocyanin contents were low compared to previous studies (8, 9, 10, 12), which seems to be influenced by adjusting pH and sterilization during media production. However, despite the low anthocyanin contents, antioxidant and antiproliferative activities in fermented blueberry extracts appear to be due to the individual or synergistic effects of changed phenolic compounds. In conclusion, our results suggest that fermented blueberries can increase antioxidant activities and inhibit the proliferation of human cervical carcinoma HeLa cells and can be applied in the food industry. Furthermore, studies on antioxidant and antiproliferative activities of increased compounds by fermentation in blueberries are needed.

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