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# Combined Effects of Probiotic Fermentation and High-Pressure Extraction on the Antioxidant, Antimicrobial, and Antimutagenic Activities of Deodeok (*Codonopsis lanceolata*)

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This study was designed to evaluate the combined effects of probiotic fermentation and high-pressure extraction (HPE) on the functional properties of *Codonopsis lanceolata*. The ground *C. lanceolata* samples were anaerobically fermented with *Lactobacillus acidophilus* ADH, *Bifidobacterium longum* B6, *Lactobacillus rhamnosus* GG, or *Lactobacillus paracasei* at 37 °C for 10 days and subjected to 500 MPa at 50 °C for 30 min. The extraction yields of *C. lanceolata* samples were noticeably increased to 29−32% by HPE. The *B. longum*-fermented *C. lanceolata* samples extracted by high pressure (BLF-HPE) exhibited the highest antimicrobial activity (MIC < 14 mg/mL) against *Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella boydii*, and *Salmonella typhimurium*. The nonfermented *C. lanceolata* samples extracted with high pressure (NF-HPE) had the highest total phenolic content (13.3 mg of GAE/g). The lowest effective concentrations ($EC_{50}$ and $EC_{0.5}$) were 4.55 and 1.76 mg/mL, respectively, for NF-HPE extracts, indicating its highest antioxidant activity. The BLF-HPE and *L. rhamnosus*-fermented *C. lanceolata* samples extracted by high pressure (LRF-HPE) exhibited the highest antimutagenic activities in *S. typhimurium* TA 100, which were 82 and 83% inhibition, respectively. The use of probiotic fermentation and HPE can produce more biologically active compounds in *C. lanceolata* than the conventional solvent extraction method. The results provide pharmaceutically useful information for improving biological properties and an approach to drug discovery.

**KEYWORDS: *Codonopsis lanceolata*; deodeok; high pressure; probiotic fermentation; antioxidant; antimicrobial; antimutagenicity**

## INTRODUCTION

*Codonopsis lanceolata* (Bench et Hook), a perennial flowering herb, belongs to the Campanulaceae family and has long been used as traditional folk medicine to treat asthma, phthisis, tuberculosis, bronchitis, antitussive, expectorant, antidote, dyspepsia, and psychoneurosis in China, Japan, and Korea (*1*−*3*). It has also been used in Korean cuisines, called deodeok. Over the past few decades, deodeok has attracted a growing amount of attention because of its pharmacological effects such as antimutagenic, antifatigue, anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory activities (*4*−*6*). The biological activities of deodeok are attributed to the various constituents, including polyphenols, saponins, tannins, alkaloids, steroids, and essential oils (*1*−*3*, *7*). Thus, *C. lanceolata* can possibly be used as a less costly alternative to *Panax ginseng* which is well-known as the adaptogenic herb (*8*). However, there are relatively few studies focused on enhancing pharmacological activities in *C. lanceolata*.

Fermentation is commonly used to break down certain undesirable compounds, induce effective microbial conversion, and improve potential nutraceutical values (*9*, *10*). Previous studies have reported that the fermentation process could modify naturally occurring constituents, including isoflavons, saponins, phytosterols, and phenols, and enhance biological activities, specifically antioxidant and antimicrobial properties (*9*, *11*, *12*). Probiotic strains used for fermentation exert beneficial effects and are safe (*13*). In recent years, since extraction has played a critical role in purifying active ingredients from raw plant sources, novel technologies such as pulsed electric fields, ultrasonication, supercritical fluid, microwave, and high pressure have been applied to increase the efficacy in the extraction of herbs and plant materials (*14*, *15*). These applications of novel extractions over

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traditional solvent−thermal extractions can help further release remaining bioactive compounds. In particular, high-pressure extraction (HPE) is of great interest because it can effectively extract phytochemicals with a less negative impact on their biological functions (*16*). HPE not only improves the extraction efficacy but also reduces the extraction time (*14*, *17*). Therefore, the objective of this study was to investigate the combined effects of probiotic fermentation and HPE in enhancing the antimicrobial, antioxidant, and antimutagenic activities of *C. lanceolata*.

## MATERIALS AND METHODS

**Plant Materials and Reagents.** The roots of *C. lanceolata* (Bench et Hook) were obtained from a local market (Hoengseong, Gangwon, Korea). The *C. lanceolata* samples were dried using a cabinet-type convective dryer at 50 °C for 5 days and ground with a high-speed grinder (SFM-555SP, Shinil Industrial Co., Ltd., Seoul, Korea). The ground samples were used for probiotic fermentation. Phenolic compounds (vanillic acid, vanillin, *p*-coumaric acid, *p*-hydroxybenzoic acid, *trans*-cinnamic acid, *p*-hydroxybenzaldehyde, and *trans*-ferulic acid), Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, methanol, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was purchased from Merck KGaA (Darmstadt, Germany). HPLC grade water was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). All chemicals and solvents were HPLC or analytical grade.

**Bacterial Strains and Culture Conditions.** Strains of *Lactobacillus acidophilus* ADH, *Bifidobacterium longum* B6, *Lactobacillus rhamnosus* GG, and *Lactobacillus paracasei* (ATCC 25598) were anaerobically cultivated in De Man, Rogosa, and Sharpe (MRS) (BD, Becton, Dickinson and Co., Sparks, MD) broth supplemented with 0.05% cysteine hydrochloride at 37 °C for 24 h. Strains of *Listeria monocytogenes* (KACC 12671), *Staphylococcus aureus* (KACC 10196), *Shigella boydii* (KACC 10792), and *Salmonella typhimurium* (KCCM 40253) obtained from the Korean Agricultural Culture Collection (KACC, Suwon, Korea) and the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea) were used as indicators for antimicrobial susceptibility assays. The strains were cultivated aerobically in trypticase soy broth (TSB) (BD) at 37 °C for 20 h. After cultivation, cultures were harvested at 3000*g* for 20 min at 4 °C. The harvested cells were diluted to approximately $10^8$ colony forming units (CFU)/mL in 0.1% sterile buffered peptone water (BPW) for inoculation. The *S. typhimurium* tester strains (TA 98, TA 100, TA 1535, and TA 1537) for the Ames mutagenicity assay were purchased from the Korea Institute of Toxicology (KIT, Daejeon, Korea) and cultured in nutrient broth (NB) (BD) at 37 °C for 18 h. The genotype patterns of the tester strains were confirmed according to the instruction provided by the KIT (*18*).

**Probiotic Fermentation.** The ground *C. lanceolata* samples (100 g each) were aseptically mixed with 250 mL of sterile distilled water and 180 mL of MRS broth. The mixtures were aseptically inoculated with approximately $10^6$ CFU/g of each probiotic, *L. acidophilus*, *B. longum*, *L. rhamnosus*, or *L. paracasei*. Each probiotic culture-inoculated sample or noninoculated sample (control) was fermented at 37 °C for 10 days in a GasPak anaerobic system (BBL, Cockeysville, MD) with Anaerogen (Oxoid, Ltd., Basingstoke, Hampshire, U.K.). All experiments were conducted with three replicates. The fermented samples were tested for determination of microbial numbers and pH on days 0 and 10.

**Microbiological Analysis.** The fermented samples (1 g each) were serially (1:10) diluted with 0.1% BPW and pour-plated with MRS agar. The agar plates were incubated at 37 °C for 48 h to enumerate the population of probiotic strains.

**pH Measurement.** Each fermented sample was directly used to measure the pH after fermentation for 0 and 10 days using a pH meter (Fisher Scientific, St. Louis, MO).

**High-Pressure Extraction.** For high-pressure extraction (HPE), the fermented and nonfermented roots of *C. lanceolata* were mixed with 70% ethanol and subjected to 500 MPa at 50 °C for 30 min. For the conventional extraction, the nonfermented roots of *C. lanceolata* were extracted with 70% ethanol at 80 °C for 24 h. After extraction, the samples were filtered, concentrated using a rotary vacuum evaporator at 60 °C, lyophilized in a freeze-dryer, and stored at −20 °C prior to analyses of phenolic compounds, DPPH assay, antimicrobial activity, and mutagenicity test. The extraction yields were calculated as compared to the crude samples.

**Determination of Antibacterial Activity.** A broth microdilution method with slight modifications of the Clinical Laboratory Standards Institute (CLSI) procedure (*19*) was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *C. lanceolata* extracts. The extracts were dissolved and sterilized by filtration using a 0.20 $\mu$m filter. The sterilized extracts were serially diluted to concentrations ranging from 10 to 500 mg/mL with TSB in 96-well plates. The TSB medium was used as a negative control. The initial populations of indicator strains, *L. monocytogenes*, *St. aureus*, *Sh. boydii*, and *S. typhimurium*, were approximately $1.70 \times 10^5$ CFU/mL in each well. All inoculated plates were cultured for 20 h at 37 °C. Viable counts were determined by the pour-plate technique using TSA. The MIC (minimum inhibitory concentration) is the lowest concentration (milligrams per milliliter) of *C. lanceolata* extracts at which bacterial growth is reduced by more than 99%. The MBC (minimum bactericidal concentration) is the lowest concentration (milligrams per milliliter) of *C. lanceolata* extracts at which bacterial growth is inhibited by more than 99.9%. The dose−response curves for bacterial growth were analyzed to determine the antibacterial parameters (MIC and MBC) using the Nonlinear Curve Fitting Function of Microcal Origin version 7.5 (Microcal Software Inc., Northampton, MA).

**Determination of Levels of Total Phenols and Flavonoids.** The total phenolic compound contents of extracts were determined using the Folin-Ciocalteu assay (*20*) with slight modifications. Each extract (0.1 g) was diluted to 1 mL with distilled water. The diluted samples were mixed with 1 mL of diluted (1:10) Folin-Ciocalteu reagent and incubated at 22 °C for 5 min. The mixtures were reacted with 1 mL of a 10% sodium carbonate solution and allowed to stand at 22 °C for 1 h. The absorbance was measured using a microplate reader at 760 nm. A standard curve was prepared at 0.5, 1.0, 1.5, 2.5, 5.0, 7.5, and 10.0 mg/mL gallic acid in the same manner as described for the extracted samples. Total phenolic concentrations were expressed as milligrams of gallic acid equivalent (GAE) per gram of extract. The flavonoid content was determined using a spectrometric method (*21*). Each extract (0.5 mL) was serially mixed with 0.1 mL of 10% aluminum nitrate, 0.1 mL of 1 M aqueous potassium acetate, and 4.3 mL of ethanol. The mixture was allowed to stand at room temperature for 40 min, and then the absorbance was measured spectrophotometrically at 415 nm. A standard curve was prepared at 0, 2, 4, 6, 8, and 10 mg/mL quercetin as described above. Flavonoid contents were expressed as milligrams of quercetin equivalent (QE) per gram of extract.

**HPLC Analysis of Phenolic Acids.** Phenolic monomers and dimers were analyzed using the high-performance liquid chromatography (HPLC, Waters M600E, Milfold, MA) system equipped with a UV absorbance detector (280 nm, 0.05 AUFS). The separations of free phenolic acids were achieved in the reverse phase mode using a Waters Spherisorb ODS2 column [250 mm × 4.6 mm (inside diameter), 5 $\mu$m]. The mobile phases were solvent A [1 mM trifluoroacetic acid (TFA) in 10% (v/v) acetonitrile] and solvent B [1 mM TFA in 40% (v/v) methanol and 40% (v/v) acetonitrile]. The gradient profile was as follows (*22*): 90% A and 10% B (initial), 90% A and 10% B from 0 to 10 min, 60% A and 40% B from 10 to 15 min, 60% A and 40% B from 15 to 24 min, 0% A, 5% B, and 100% C from 24 to 40 min, 90% A, 10% B, and 100% C from 40 to 45 min, and 90% A, 10% B, and 100% C from 45 to 50 min at a flow rate of 1 mL/min. Peaks were identified by retention times established from standard solutions. Pure phenolic acid standards (*p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, coumaric acid, *trans*-ferulic acid, and *trans*-cinnamic acid) were dissolved in methanol and serially diluted to 10, 50, or 100 mg/mL. Dilutions and peak areas were used to establish standard curves.

**DPPH Radical Scavenging Assay.** The antioxidant activity of the fermented samples and control was determined with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (*23*). Two milliliters of the extracted sample (1%, w/v) was mixed with 1 mL of a 0.2 mM DPPH radical solution in 95% ethanol. The mixture was incubated at 25 °C for 30 min and the absorbance at 517 nm measured. The scavenging activity of the DPPH radical was calculated by the following equation:

$$\text{DPPH scavenging activity (\%)} = (1 - A_t/A_c) \times 100$$

where $A_c$ is the absorbance of the control reaction and $A_t$ is the absorbance of the extract.

The efficient concentration ($EC_{50}$) was estimated from the percentage of DPPH scavenging activity plotted as a function of the concentration of *C. lanceolata* extract, which was expressed in terms of the concentration (milligrams per milliliter) required for 50% reduction of DPPH.

**Ferric Reducing Power Assay.** A spectrometric method of Oyaizu (*24*) with a slight modification was used to determine the reducing power of extracts. Each extract (1 mL) was mixed with 0.2 M sodium phosphate buffer (1 mL, pH 6.6) and 1% aqueous potassium ferricyanide (1 mL). The mixture was placed in a water bath at 50 °C for 20 min, immediately cooled to room temperature, and mixed with 1 mL of 15% trichloroacetic acid, which was centrifuged at 1500*g* for 15 min. The collected supernatant (1 mL) was diluted with 1 mL of distilled water and then mixed with 1 mL of 0.1% ferric chloride to reduce $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$. The absorbance was measured spectrophotometrically at 700 nm. The efficient concentration ($EC_{0.5}$) was estimated from the absorbance plotted as a function of the concentration of *C. lanceolata* extract, denoting the concentration (milligrams per milliliter) required to achieve an absorbance of 0.5.

**Ames *Salmonella* Mutagenicity Assay.** The mutagenic activity of *C. lanceolata* extracts was evaluated using the plate incorporation test described by Maron and Ames (*18*). The extracts were dissolved in distilled water to final concentrations of 2.5, 5, 25, and 50 mg/plate. A rat liver S9 mixture was used as an exogenous mammalian metabolic activation system. The negative control was water for the four tester strains. Positive controls were 4-nitro-*o*-phenylenediamine (2.5 μg/plate) for TA 98, sodium azide (5.0 μg/plate) for TA 100 and TA 1535, and 9-aminoacridine (50 μg/plate) for TA 1537 without metabolic activation. In the presence of exogenous metabolic activation (S9), 2-aminoanthracene (5.0 μg/plate) was used as a positive mutagen for all tester strains. The extracted sample (0.05 mL of *C. lanceolata* extract or control) and the histidine-dependent *S. typhimurium* strain (0.05 mL: TA 98, TA 100, TA 1535, or TA 1537) gently were mixed with or without the S9 mixture (0.5 mL) containing NADPH in 2 mL of top agar containing biotin and histidine at 37 °C for 20 min. The mixture was pour-plated on glucose minimal (GM) agar, and the plates were incubated at 37 °C. The numbers of $His^+$ revertants on the test plates were determined after incubation for 48 h.

**Antimutagenicity Assay.** *C. lanceolata* extract (50 mg), positive mutagen (0.05 mL), and *S. typhimurium* strain TA 98, TA 100, TA 1535, or TA 1537 were mixed with the S9 mixture (0.5 mL). The mixture was pour-plated on glucose minimal (GM) agar, and the plates were incubated at 37 °C. The numbers of revertant colonies on the plates were determined after incubation for 48 h at 37 °C. The antimutagenic potential (AMP, %) was calculated as follows:

$$\mathrm{AMP} = \frac{N_P - N_M}{N_P - N_N} \times 100$$

where $N_P$, $N_M$, and $N_N$ are the numbers of mutagen-induced, mixture-induced, and spontaneous revertants, respectively.

**Statistical Analysis.** All experiments were performed in duplicate for three replicates. Data were analyzed using Statistical Analysis System (SAS). The General Linear Model (GLM) and least significant difference (LSD) procedures were used to compare means among treatments at $p < 0.05$.

## RESULTS AND DISCUSSION

The viable counts and pH changes in *C. lanceolata* samples were observed during fermentation as shown in **Table 1**. The viable counts in probiotic fermented *C. lanceolata* samples increased up to 7−9 log CFU/g. *B. longum* B6 (BLF)- and *L. rhamnosus* GG (LRF)-fermented *C. lanceolata* samples had the highest populations, while the lowest population was observed in *L. acidophilus* ADH (LAF). The results indicate that *C. lanceolata* samples provided good substrates for probiotic growth, except for *L. acidophilus*. The pH values in all fermented *C. lanceolata* samples decreased to approximately 3−4 after fermentation for 10 days. The extent of pH decreases during fermentation corresponded to that of the probiotic growth in *C. lanceolata* samples. This suggests that all probiotic strains used produced organic acids during the fermentation process.

**Table 1.** Viable Counts of Probiotic Strains and Changes in pH during the Fermentation of *C. lanceolata*

| | viability (log CFU/g)[b] | | pH[b] | |
|---|---|---|---|---|
| treatment[a] | day 0 | day 10 | day 0 | day 10 |
| NF | ND[c] | ND[c] | 5.40 ± 0.07 a | 5.26 ± 0.18 a |
| BLF | 6.22 ± 0.21 a | 9.04 ± 0.18 a | 5.39 ± 0.11 a | 3.26 ± 0.17 c |
| LAF | 6.39 ± 0.06 a | 7.01 ± 0.12 c | 5.40 ± 0.01 a | 4.05 ± 0.21 b |
| LPF | 6.37 ± 0.24 a | 8.10 ± 0.15 b | 5.42 ± 0.10 a | 3.30 ± 0.12 c |
| LRF | 6.24 ± 0.32 a | 9.12 ± 0.29 a | 5.43 ± 0.03 a | 3.25 ± 0.08 c |

[a] NF, BLF, LAF, LPF, and LRF stand for nonfermentation and *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG fermentation, respectively ($n = 6$). [b] Means with different letters within a column are significantly different at $p < 0.05$. [c] ND means that the number of viable probiotic bacteria is below the detection limit (1 log CFU/g).

**Table 3.** Effective Concentrations (milligrams per milliliter) of *C. lanceolata* Extracts in DPPH Scavenging[a] and Reducing Power Assays[a]

| treatment[b] | DPPH ($EC_{50}$)[c] | reducing power ($EC_{0.5}$)[d] |
|---|---|---|
| NF-CE | 7.18 ± 0.68 a | 2.42 ± 0.20 ab |
| NF-HPE | 4.55 ± 0.47 d | 1.76 ± 0.17 e |
| BLF-HPE | 5.18 ± 0.30 d | 2.34 ± 0.27 bc |
| LAF-HPE | 7.09 ± 0.17 a | 2.51 ± 0.23 a |
| LPF-HPE | 6.43 ± 0.31 b | 2.26 ± 0.14 cd |
| LRF-HPE | 5.58 ± 0.53 d | 2.15 ± 0.12 d |

[a] Means with different letters within a column are significantly different at $p < 0.05$ ($n = 6$). [b] NF-CE, NF-HPE, BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE stand for nonfermentation by conventional extraction and *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG fermentation followed by high-pressure extraction, respectively. [c] The $EC_{50}$ represents the effective concentration required to scavenge DPPH radicals by 50%. [d] The $EC_{0.5}$ represents the effective concentration required to achieve an absorbance of 0.5.

**Table 2.** Antimicrobial Activities of the Extracts of *C. lanceolata* against Selected Food-Borne Pathogens

| | *L. monocytogenes* | | *St. aureus* | | *Sh. boydii* | | *S. typhimurium* | |
|---|---|---|---|---|---|---|---|---|
| treatment[a] | MIC[b] | MBC[c] | MIC[b] | MBC[c] | MIC[b] | MBC[c] | MIC[b] | MBC[c] |
| NF-CE | >500 | >500 | >500 | >500 | 447 ± 28 | >500 | 463 ± 18 | >500 |
| NF-HPE | 209 ± 8 | >500 | 327 ± 6 | >500 | 265 ± 35 | >500 | 301 ± 4 | >500 |
| BLF-HPE | 14 ± 3 | 28 ± 2 | 12 ± 1 | 23 ± 1 | <10 | 24 ± 3 | <10 | 18 ± 1 |
| LAF-HPE | 62 ± 2 | 82 ± 3 | 45 ± 2 | 68 ± 4 | 57 ± 3 | 75 ± 2 | 57 ± 2 | 74 ± 4 |
| LPF-HPE | 12 ± 1 | 28 ± 1 | 12 ± 1 | 39 ± 2 | 12 ± 2 | 27 ± 1 | 18 ± 4 | 30 ± 1 |
| LRF-HPE | 13 ± 2 | 36 ± 3 | 12 ± 4 | 37 ± 1 | <10 | 26 ± 1 | <10 | 20 ± 2 |

[a] NF-CE, NF-HPE, BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE stand for nonfermentation by conventional extraction and *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG fermentation followed by high pressure extraction, respectively ($n = 6$). [b] The MIC (minimum inhibitory concentration) is the lowest concentration (milligrams per milliliter) at which bacterial growth is reduced by more than 99%. [c] The MBC (minimum bactericidal concentration) is the lowest concentration (milligrams per milliliter) at which bacterial growth is inhibited by more than 99.9%.

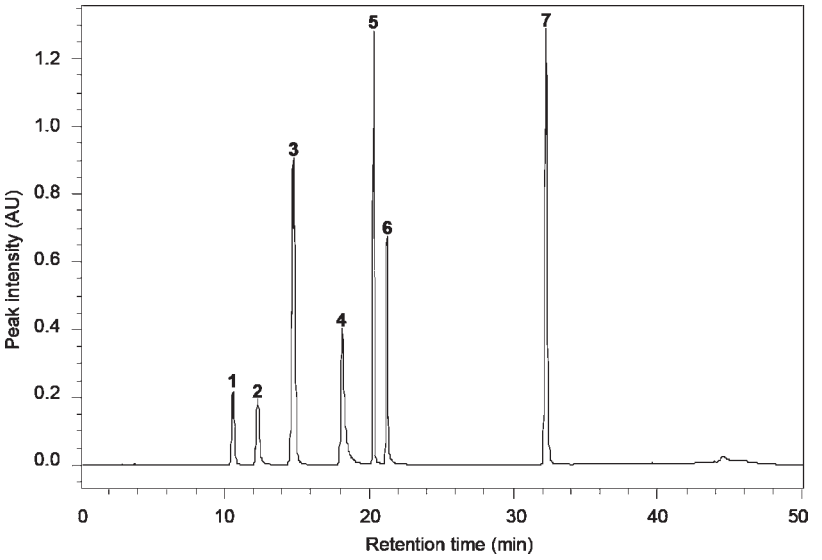


**Figure 1.** Typical HPLC chromatogram of phenolic acid standards: (1) *p*-hydroxybenzoic acid, (2) vanillic acid, (3) *p*-hydroxybenzaldehyde, (4) vanillin, (5) *trans-p*-coumaric acid, (6) *trans*-ferulic acid, and (7) *trans*-cinnamic acid.

Nonfermented *C. lanceolata* extracted by conventional extraction using ethanol (NF-CE) showed the lowest extraction yield (21.76 ± 0.83%). In this study, ethanol extraction was used as a conventional extraction method to compare with high-pressure extraction (HPE) because it has been reported to increase the solubility of flavonoids, phenolic acids, and anthocyanins (*15*). The HPE significantly increased the extraction yield when compared to the NF-CE. According to Ahmed and Ramaswamy (*25*), the improved extraction efficacy of *C. lanceolata* samples might result from the enhancement of the mass transfer rate and solvent permeability through homogeneous pressure distribution. The increased permeability might be attributed to the disruption of hydrophobic bonds and the deprotonation of charged groups (*17*). High pressure and a polar solvent (ethanol) could act synergistically to extract endocellular components such as phenolic acids, lignans, polysaccharides, and flavonoids (*15*). The highest extraction yield was obtained at the NF-HPE (32.14 ± 1.50%), followed by LRF-HPE (31.82 ± 1.71%), LAF-HPE (31.34 ± 1.33%), LP-HPE (30.82 ± 1.71%), and BL-HPE (28.51 ± 1.34%). The extraction efficacy was not significantly increased by fermentation. The results suggest that the level of fermentation increased the production of secondary metabolites, leading to a change in functional group profiles with respect to extraction properties. The coefficients of variation (CVs) were less than 0.1, indicating good reproducibility of conventional extraction and HPE.

As shown in **Table 2**, the probiotic fermented *C. lanceolata* extracted by high pressure (BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE) exerted significant antibacterial activity against *L. monocytogenes*, *St. aureus*, *Sh. boydii*, and *S. typhimurium*. The MIC values of BLF-HPE, LPF-HPE, and LRF-HPE were less than 18 mg/mL, while those of LAF-HPE were less than 62 mg/mL. *L. monocytogenes*, *St. aureus*, *Sh. boydii*, and *S. typhimurium* were more susceptible to BLF-HPE, LPF-HPE, and LRF-HPE than LAF-HPE, NF, and NF-HPE. The NF-HPE (209 mg/mL < MIC < 327 mg/mL) exhibited higher antimicrobial activity than the NF-CE (MIC > 500 mg/mL). The results suggest that active antimicrobial metabolites may be produced during the fermentation process. Three types of interactions are expected in probiotic fermented *C. lanceolata*, including additive, synergistic, and antagonistic effects. The antimicrobial activity in the probiotic fermented *C. lanceolata* extracts was increased due

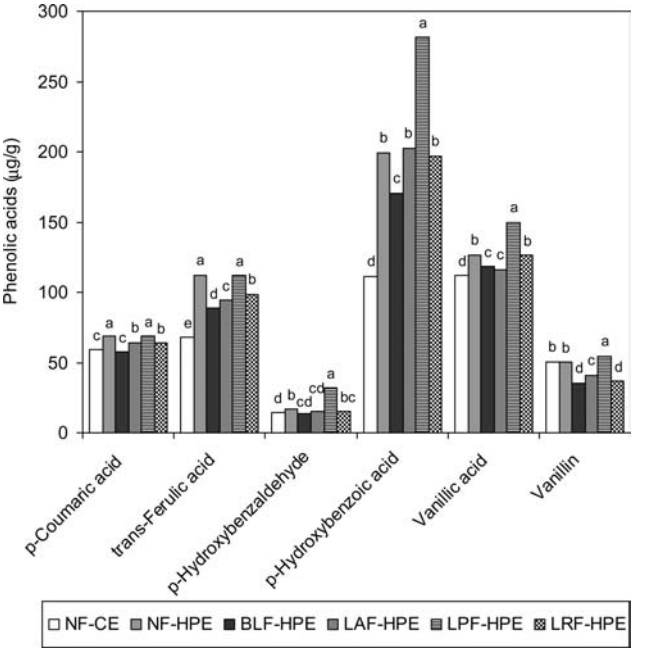


**Figure 2.** Phenolic acids (micrograms per gram) in the extracts of *C. lanceolata*: NF-CE, nonfermentation followed by conventional extraction; NF-HPE, nonfermentation followed by HPE; BLF-HPE, *B. longum* B6 fermentation followed by HPE; LAF-HPE, *L. acidophilus* ADH fermentation followed by HPE; LPF-HPE, *L. paracasei* ATCC 25598 fermentation followed by HPE; LRF-HPE, *L. rhamnosus* GG fermentation followed by HPE (values with different letters are significantly different at $p < 0.05$).

to the synergistic effects of bioactive metabolites in the presence of phytochemicals (*26*). BLF-HPE had the greatest bactericidal activity against *L. monocytogenes*, *St. aureus*, *Sh. boydii*, and *S. typhimurium*, showing MBC values between 18 and 28 mg/mL. Antimicrobial compounds have bacteriostatic and bactericidal effects depending on their concentrations and cell types, which disrupt the function of cell permeability, transport mechanisms, oxidative phosphorylation, peptidoglycan synthesis, lipopolysaccharide synthesis, and signal transduction (*27*). Because effective antimicrobials inhibit the growth of pathogens on more than one target site, a synergistic effect of the combination of fermentation

**Table 4.** Mutagenic Activities (revertants per plate) of the Extracts of *C. lanceolata* Using *S. typhimurium* Strains

| | | frame-shift type | | | | base-pair substitution type | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | TA 98 | | TA 1537 | | TA 100 | | TA 1535 | |
| treatment[a] | concn (mg/plate) | without S9 | with S9 | without S9 | with S9 | without S9 | with S9 | without S9 | with S9 |
| negative | | 49 | 54 | 10 | 8 | 180 | 170 | 22 | 19 |
| NF-CE | 2.5 | 55 | 61 | 13 | 9 | 191 | 165 | 22 | 21 |
| | 5 | 49 | 45 | 11 | 9 | 196 | 194 | 19 | 19 |
| | 25 | 65 | 61 | 10 | 11 | 217 | 166 | 18 | 20 |
| | 50 | 74 | 82 | 21 | 9 | 268 | 211 | 24 | 17 |
| NF-HPE | 2.5 | 43 | 38 | 9 | 11 | 209 | 164 | 18 | 16 |
| | 5 | 50 | 46 | 10 | 7 | 198 | 204 | 18 | 20 |
| | 25 | 56 | 60 | 11 | 11 | 198 | 201 | 27 | 16 |
| | 50 | 55 | 70 | 8 | 6 | 229 | 203 | 16 | 16 |
| BLF-HPE | 2.5 | 30 | 33 | 9 | 7 | 212 | 164 | 20 | 18 |
| | 5 | 35 | 38 | 13 | 7 | 268 | 195 | 25 | 18 |
| | 25 | 27 | 35 | 10 | 9 | 198 | 171 | 16 | 17 |
| | 50 | 0 | 41 | 0 | 0 | 244 | 0 | 0 | 18 |
| LAF-HPE | 2.5 | 35 | 43 | 7 | 8 | 188 | 182 | 16 | 13 |
| | 5 | 58 | 58 | 9 | 7 | 174 | 163 | 28 | 20 |
| | 25 | 65 | 54 | 10 | 10 | 262 | 180 | 23 | 18 |
| | 50 | 44 | 69 | 15 | 13 | 296 | 212 | 19 | 22 |
| LPF-HPE | 2.5 | 39 | 43 | 8 | 8 | 195 | 178 | 18 | 14 |
| | 5 | 29 | 37 | 12 | 7 | 182 | 189 | 22 | 20 |
| | 25 | 33 | 36 | 11 | 7 | 183 | 128 | 18 | 17 |
| | 50 | 56 | 32 | 8 | 14 | 156 | 0 | 25 | 25 |
| LRF-HPE | 2.5 | 45 | 48 | 9 | 11 | 205 | 192 | 20 | 14 |
| | 5 | 40 | 44 | 12 | 10 | 233 | 186 | 19 | 21 |
| | 25 | 29 | 31 | 7 | 8 | 116 | 136 | 23 | 23 |
| | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| positive | | 526 | 633 | 210 | 193 | 1768 | 720 | 770 | 305 |

[a] The negative control was sterile distilled water. NF-CE, NF-HPE, BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE stand for nonfermentation by conventional extraction and *B. longum* B6, *L. acidophilus* ADH, *L. paracasei* ATCC 25598, and *L. rhamnosus* GG fermentation followed by HPE, respectively. Positive controls are 4-nitro-*o*-phenylenediamine (2.5 $\mu$g/plate), 9-aminoacridine (50 $\mu$g/plate), sodium azide (5.0 $\mu$g/plate), and sodium azide (5.0 $\mu$g/plate) for TA 98, TA 1537, TA 100, and TA 1535, respectively, in the absence of S9. 2-Aminoanthracene (5.0 $\mu$g/plate) is used to induce the revertant colonies for all tester strains in the presence of S9.

and HPE may improve the antimicrobial effectiveness of *C. lanceolata* extracts.

HPE significantly increased the total phenolic content in *C. lanceolata* samples compared to NF-CE. The highest phenolic content in *C. lanceolata* samples was observed from the NF-HPE (13.32 ± 0.47 mg of GAE/g), followed by LR-HPE (13.24 ± 0.54 mg of GAE/g), LP-HPE (12.29 ± 0.48 mg of GAE/g), and BL-HPE (12.01 ± 0.65 mg of GAE/g). The NF-HPE and BL-HPE extracts showed the highest flavonoid contents of 6.50 and 6.63 mg of QE/g, respectively. However, there was no significant difference in flavonoid contents among NF-CE, LA-HPE, LP-HPE, and LR-HPE extracts. Antioxidant activity is primarily associated with the content of polyphenolic components, glycosides, and flavonoids (*28*). Phenolic compounds are classified as primary or chain-breaking antioxidants on the basis of their mechanisms of antioxidant activities, including electron or hydrogen donating, metal chelating, free radical scavenging, and lipoxygenase inhibiting abilities (*29*). The DPPH scavenging activity and reducing power increased with an increase in the concentration of *C. lanceolata* extracts. The highest scavenging effect and reducing power were 80% and 2.38, respectively, at 12.5 mg/mL NF-HPE extract (data not shown). The $EC_{50}$ for the DPPH scavenging effect and the $EC_{0.5}$ for the reducing power are listed in **Table 3**. NF-HPE, BLF-HPE, and LRF-HPE exhibited the highest antioxidant properties, having the lowest $EC_{50}$ and $EC_{0.5}$ values. The radical scavenging capacity and reducing power potential of *C. lanceolata* extracts were positively correlated with the content of total phenolic compounds.

Vanillic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillin, *trans*-*p*-coumaric acid, *trans*-ferulic acid, and *trans*-cinnamic acid were used for phenolic acid quantification. The representative HPLC profile of selected phenolic standards is shown in **Figure 1**. The total numbers of HPLC peaks in NF-CE, NF-HPE, BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE were 79, 84, 88, 88, 88, and 86, respectively. The number of common peaks shared by all extracts was 65. Compared to the NF-CE, the numbers of specific peaks in BLF-HPE, LAF-HPE, LPF-HPE, and LRF-HPE were 14, 15, 16, and 15, respectively. These observations suggest that different metabolites were produced during the fermentation process. Flavonoids such as quercetin, rutin, and kaempferol are degraded to phenolic acids throughout bacterial growth (*30*), and saponins are hydrolyzed to sapogenins and sugars (*31*), which may result in a significant increase in the levels of various secondary metabolites in *C. lanceolata* during the fermentation process. According to previous reports (*10*, *32*, *33*), ferulic acid and coumaric acid were further metabolized to 4-vinyl (or ethyl) phenol and 4-vinyl (or ethyl) guaiacol by the decarboxylative activity of microbial-produced hydrolytic enzymes. The contents of phenolic acids in *C. lanceolata* extracts are shown in **Figure 2**. The largest amounts of *p*-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillic acid, and vanillin were observed in the LPF-HPE at levels of 69, 32, 282, 150, and 55 $\mu$g/g, respectively. The highest content of *trans*-ferulic acid was 112 $\mu$g/g in both NF-HPE and LPF-HPE. As compared to the NF-CE, the amounts of *trans*-ferulic acid, *p*-hydroxybenzoic acid, and vanillic acid were significantly high in all extracts by HPE, indicating high pressure

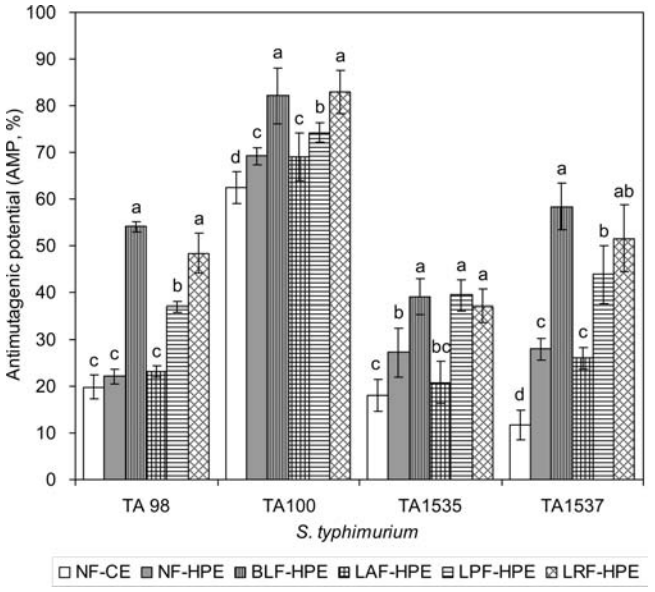


**Figure 3.** Antimutagenic effects of the extracts of *C. lanceolata* fermented by probiotic strains followed by HPE against frame-shift mutants (TA 98 and TA 1537) and base-pair substitution-carrying mutants (TA 100 and TA 1535) of *S. typhimurium* with S9: NF-CE, nonfermentation followed by conventional extraction; NF-HPE, nonfermentation followed by HPE; BLF-HPE, *B. longum* B6 fermentation followed by HPE; LAF-HPE, *L. acidophilus* ADH fermentation followed by HPE; LPF-HPE, *L. paracasei* ATCC 25598 fermentation followed by HPE; LRF-HPE, *L. rhamnosus* GG fermentation followed by HPE (values with different letters are significantly different at $p < 0.05$).

efficiently extracts and recovers phenolic acids, specifically the hydroxybenzoic acids present in bound form in *C. lanceolata*. Phenolic acids are classified as hydroxybenzoic acids (salicylic, gallic, and vanillic acids) and hydroxycinnamic acids (caffeic, chlorogenic, sinapic, fertaric, coumaric, and ferulic acids), which are responsible for sensory quality, antioxidant activity, and other physiological benefits (*33*−*35*). The hydroxycinnamic acids are known to be more effective antioxidants than the hydroxybenzoic acids (*36*).

The mutagenic activity of *C. lanceolata* extracts was evaluated against frame-shift mutants (TA 98 and TA 1537) and base-pair substitution-carrying mutants (TA 100 and TA 1535) of *S. typhimurium* with and without metabolic activation (**Table 4**). The number of spontaneous revertant colonies ranged from 8 to 180 for all test strains of TA 98, TA 100, TA 1535, and TA 1537 with and without S9, while those of mutagen-induced revertants ranged from 210 to 1768. No significant differences in the frequencies of revertants of *S. typhimurium* TA 98, TA 100, TA 1535, and TA 1537 were observed between negative controls and *C. lanceolata* extracts, indicating that all *C. lanceolata* extracts are not mutagenic against all test strains in the absence and presence of S9. The results imply that the fermentation process was not involved in the production of mutagenic metabolites and the secondary metabolites and released substances did not cause genotoxicity in vitro. However, the growth of *S. typhimurium* TA 98, TA 100, TA 1535, and TA 1537 was inhibited at high concentrations (50 mg/plate) of BLF-HPE and LRF-HPE. The extracts (BLF-HPE, LPF-HPE, and LRF-HPE) showed the highest antimutagenic activities against positive mutagen (2-aminoanthracene) in TA 98, TA 100, TA 1537, and TA 1535 of *S. typhimurium* with S9 (**Figure 3**). LRF-HPE significantly most effectively reduced mutagenicity by 83% in *S. typhimurium* TA 100, followed by BLF-HPE (82%) and LPF-HPE (74%). The results indicate that the substances metabolized and released from *C. lanceolata* during fermentation and HPE can effectively suppress the mutagen-induced mutations. The antimutagenic activity is associated with the content of *p*-coumaric acid, gallic acid, ferulic acid, caffeic acid, syringic acid, vanillic acid, gentisic acid, and saponins (*37*−*39*).

In conclusion, the fermentation process combined with high-pressure extraction (HPE) revealed positive effects on antimicrobial, antioxidant, and antimutagenic activities in *C. lanceolata*. The application of probiotic fermentation and high pressure enhanced the levels of bioactive compounds in *C. lanceolata* and increased the extraction efficacy with significant advantages over the conventional solvent extraction method. Therefore, the probiotic fermentation combined with HPE could be used as an alternative extraction technique for improving biological properties of medicinal plants and also as a tool for drug discovery and development. Further studies on characterizing secondary metabolites and identifying novel bioactive substances produced during the fermentation process and HPE are currently under investigation in our laboratory.

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