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Research Article

A study on antioxidative components and activity of fermented *Cirsium Lineare (Thunb.)* extract

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Abstract This study was conducted to measure changes in polyphenol components and antioxidant effects of *Cirsium Lineare (Thunb.)* after fermentation by lactic acid bacteria. First, *Cirsium Lineare (Thunb.)* extract (CE, unfermented) and *Cirsium Lineare (Thunb.)* extract fermented with *Lactobacillus paracasei* (FCE) were prepared. Changes in components resulting from fermentation were confirmed through changes in polyphenol compound content and silymarin derivative pattern, and antioxidant activity was confirmed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, and ferric reducing antioxidant power (FRAP) analyses. As a result, polyphenol contents of CE and FCE were confirmed as 21.94 ± 1.15 and 67.90 ± 4.48 mg GAE/g, respectively. Both values were increased approximately three times by fermentation, and there was also a change in the silymarin derivative pattern. In the case of DPPH radical RC_{50} values in particular, CE and FCE were confirmed to inhibit DPPH radicals by 50% at concentrations of 129.44 ± 5.85 and 50.00 ± 3.47 μg/mL, respectively, with the FCE value approximately 2.5 times lower than that of CE. In addition, ABTS radical scavenging and FRAP activity were confirmed to share similar trends as DPPH radical scavenging activity. When CE and FCE were compared, FCE showed a better antioxidant effect overall. In conclusion, this study suggested that FCE prepared through lactic acid bacteria fermentation may be utilized as a powerful antioxidant material.

Keywords fermentation, *Lactobacillus paracasei, Cirsium Lineare (Thunb.)*, antioxidant, silymarin derivative

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Introduction

Recently, as healthy eating habits are gradually becoming a daily life, there is a lot of interest in the search and development of natural plants with excellent efficacy. In the case of plants that grow in nature, it is believed that they contain a large amount of physiologically active substances to protect themselves from the environment, so various studies on native plant resources used for food and medicine, including general vegetables, are being actively conducted.

Cirsium Lineare (Thunb.) Sch. Bip. is a perennial plant belonging to Compositae, called thorns, and is native to the country and grows in mountains or fields. *Cirsium Lineare (Thunb.)* is distributed in Korea, China and Japan. It is known to be good for anemia, detoxification, hemostasis, tonicity and diuresis, and its roots have been used to treat adult diseases such as senile blood, hepatitis, hematemesis, hypertension, and diabetes (Ishida et al. 1987; Park et al. 2004). *Cirsium Lineare (Thunb.)* flower and leaf extracts are reported to be effective in anti-obesity (Hur and Hwang 2011) and anti-diabetes (Liao et al. 2010), and recent studies have reported that they have anti-gastritis and anti-ulcer effects (Lee et al. 2011). *Cirsium Lineare (Thunb.)* contains 78 types of flavonoids including silymarin, apigenin, luteolin, myricetin, kaempferol, pectolinarin, 5,7-dihydroxy-6,4'-dimethoxyflavone, hispidulin-7-neohesperidoside flavonoid. It has anti-inflammatory and neuroprotective effects (Hur and Hwang 2011; Lee et

al. 2011).

Among natural product extraction methods, it has been reported that lactic acid bacteria used in the fermentation process have various effects such as immunity enhancement, cancer prevention effect, and antioxidant effect. It is possible to maximize the yield of active ingredients and physiologically active components by the effect, and new components can be expected (Choi et al. 2013; Kong et al. 2008). In particular, the lactic acid bacteria fermentation process is more efficient than other process methods as it requires less fermentation time and less cost for the process (Ryu and Kwon 2012).

In this study, the content of antioxidants was determined by measuring the content of total polyphenol compounds and silymarin derivatives components of *Cirsium Lineare (Thunb.)* extract (CE) and fermented *Cirsium Lineare (Thunb.)* extract (FCE). They were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, and ferric reducing antioxidant power (FRAP) analysis. Based on these results, a comparative study was conducted on the change in antioxidant activity by fermentation.

Materials and Methods

Preparation of CE and FCE

Cirsium Lineare (Thunb.) seeds used in this study were purchased from Wontech Solution Co., Ltd. As the strain used for fermentation, *Lactobacillus paracasei* acquired from Korean collection for type cultures (KCTC, Jeongeup, Korea) was used. *L. paracasei* was activated in MRS agar (Difco, USA) medium, and inoculated to a concentration of 1.0×10^9 cfu/mL in 10 mL of Nutrient broth and MRS broth, and cultured at 37°C and 160 rpm in a shaking incubator. Extraction was carried out by mixing raw *Cirsium Lineare (Thunb.)* seeds and 70% ethanol in a ratio of 10 times (w:v) to weight, respectively. Ethanol extraction was performed by mixing the 70% ethanol and the raw *Cirsium Lineare (Thunb.)* seeds for 72 h at room temperature (RT). The supernatant and the raw material were separated, extracted three times in the same way, concentrated, and then freeze-dried and used for fermentation. For fermentation, 25% of ethanol extract and 72.55% of DW were added, and glucose (Difco) 1% as a carbon source, yeast extract (Difco) 0.3%, and soytone (Difco) 0.15% as a nitrogen source were added. The strain was mixed by adding 1% (1.0×10^9 cfu/mL). The extract mixed with the medium was fermented for 3 days at 37°C and 160 RPM in a shaking incubator. After fermentation was completed, the microbes were removed through secondary filtration by centrifugation using 0.22 μm filter paper, concentrated under reduced pressure, and then freeze-dried to obtain a powder (Fig. 1).

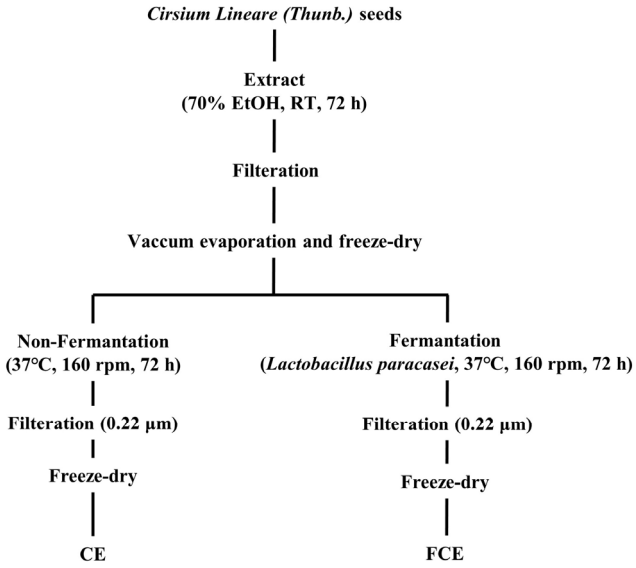

Fig. 1 Schematic diagram of CE and FCE preparation

Total polyphenolic content (TPC)

The total polyphenol content was measured according to the Folin-Denis method (Folin and Denis 1912). 60 μL of Folin reagent was added to 60 μL of the extract, mixed, and then reacted at RT for 3 min. Then, 60 μL of 10% Na_2CO_3 was added, reacted at RT for 1 h, and absorbance was measured at 700 nm. As a standard, gallic acid (Sigma, St. Louis, MO, USA) was used and the total polyphenol content was calculated from the calibration curve obtained by analyzing in the same manner as the sample. The experiment was repeated three times and the average value was presented.

High Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was performed to analyze the pattern of silymarin derivatives compounds in the CE and FCE. The sample and Silymarin were dissolved in MeOH at a concentration of 1 mg/mL, sonicated for 10 min to dissolve the sample well, and filtered through a 0.2 μm filter to prepare the sample. The HPLC system used for the analysis (Shimadzu Corporation, Kyoto, Japan) was equipped with

a CBM-20A communications bus module and SPD-M20A diode array detector. C18 (250 × 4.6 mm, 5 μm) was selected as the analytical column, and the column temperature was set to RT. As the mobile phase, 0.01% acetic acid was selected for solvent A and MeOH was selected for solvent B. The solvent composition according to time was eluted by gradient elution as follows. 0-3 min, 47% B; 3-13 min, 50% B; 13-25 min, 60% B; 25-35 min, 60% B; 35-35.01 min, 47% B; 35-45 min, 47% B was designed, and the analysis time for all samples was 45 min in total. The flow rate was set to 1.0 mL/min, and the injection volume was set to 10 μL. The analysis wavelength was measured to be 288 nm.

DPPH radical scavenging activity

The DPPH radical scavenging activity of each extract was evaluated by modifying the method of Blois (1958). 0.2 mM DPPH (Sigma) solution was added in the same ratio to each concentration sample, mixed well, and left in the dark for 30 min, and then absorbance was measured at 517 nm. The result value was expressed as a radical removal activity compared to the control group to which the sample was not added, and ascorbic acid (Sigma) was used as a positive control group. The DPPH radical scavenging activity experiment was repeated three times, and the average value was presented, and the calculation formula is as follows.

$$\text{DPPH radical scavenging (\%)} = (A - B)/A \times 100$$

A: Absorbance of the control
B: Absorbance of the sample

ABTS radical scavenging activity

When ABTS and potassium persulfate are mixed and placed in a dark place, ABTS cations are generated. The cations are removed by reacting with the antioxidant substances in the extract, and the characteristic blue-green color is discolored (Re et al. 1999). A 7.4 mM ABTS (Sigma) solution and 2.6 mM ammonium persulfate (Sigma) were mixed and reacted in the dark for about 24 h, and then diluted with phosphate buffer saline (pH 7.4) so that the absorbance at 732 nm was 0.7±0.03. 50 μL of each concentration sample was added to 950 μL of the diluted solution, mixed well, reacted at RT for 10 min, and then absorbance was measured at 732 nm. The result value was expressed as a radical scavenging activity compared to the control group to which the sample was not added, and ascorbic acid was used as a positive control group.

FRAP activity

Antioxidant activity by FRAP was measured according to the method of Benzie and Strain (1996). First, 25 mL of sodium acetate buffer (0.3 M, pH 3.6), 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ, Sigma) dissolved in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 3 mL of DW were mixed to make a mixture and maintained at 37°C until just before the experiment. CE and FCE were diluted to various concentrations and used. For the experiment, 1.5 mL of the mixture was added to 0.05 mL of the sample solution, mixed, and reacted at 37°C for 30 min, and absorbance was measured at 593 nm.

Statistical analysis

The data of all experiments were calculated as the average value of three analyzes.

For statistical significance analysis between groups, ANOVA analysis was performed using the statistical program SPSS statistics (ver. 25, IBM Co., Armonk, NY, USA). The statistical significance of the mean value was analyzed using Duncan's multiple range test at $p < 0.05$ level.

Results

Total polyphenol content of CE and FCE

The total polyphenol content of CE and FCE was calculated in terms of gallic acid equivalent. Since changes in the content of bioactive components of *Cirsium Lineare (Thunb.)* fermented with lactic acid bacteria can be a fundamental factor in increasing useful bioactivity of fermented milk thistle extract, the total phenol content of CE and FCE was compared in this experiment. As a result, the total polyphenols of CE and FCE were 21.94 ± 1.15 and 67.90 ± 4.48 mg GAE/g, respectively, which increased about three times by fermentation (Fig. 2).

Changes in the content of Silymarin derivative compounds in CE and FCE

In order to confirm the changes in the silymarin-based

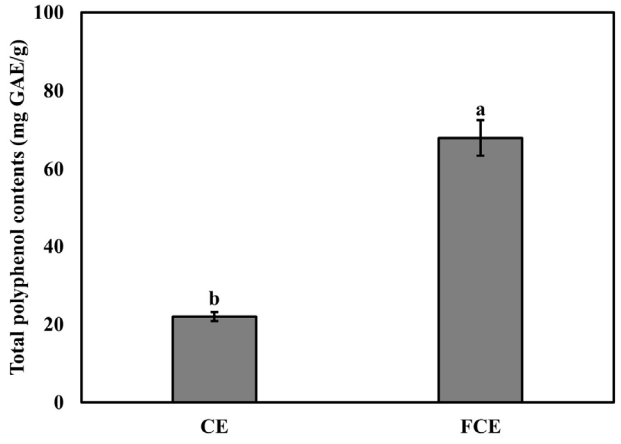

Fig. 2 Total polyphenol contents of CE and FCE. The total polyphenol content was analyzed as gallic acid equivalents mg/g of CE and FCE. Data are expressed as mean ± standard deviation (n = 3). The different letters indicate significant differences at $p < 0.05$ using Duncan's multiple range test

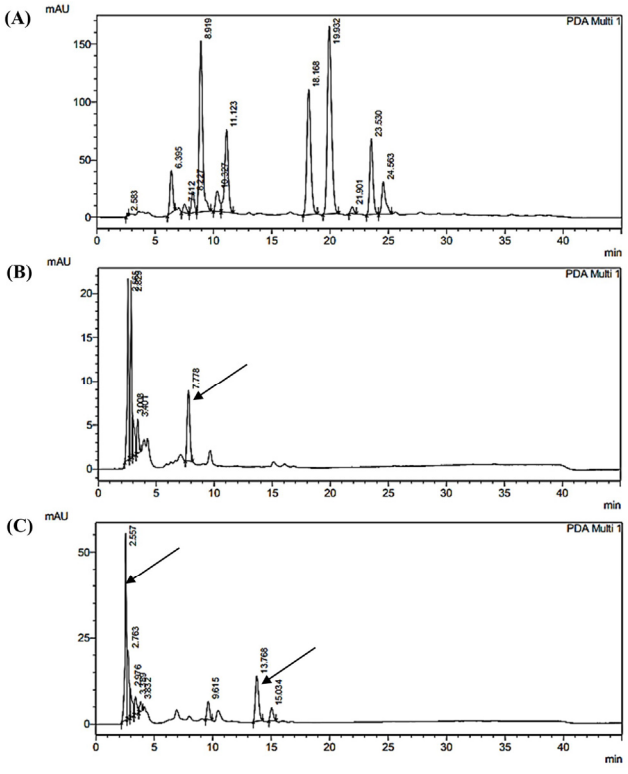

Fig. 3 Comparative chromatogram of silymarin derivatives in CE and FCE. (A) Chromatogram of silymarin (standard), (B) CE, (C) FCE

compounds of CE and FCE, HPLC was used to analyze them. As a result of the analysis, comparing the HPLC chromatogram of CE (Fig. 3(B)) and FCE (Fig. 3(C)), the peak at 7.7 min decreased in FCE, the peak increased at 2.5 min. Also, a new peak was generated at 14 min (Fig. 3).

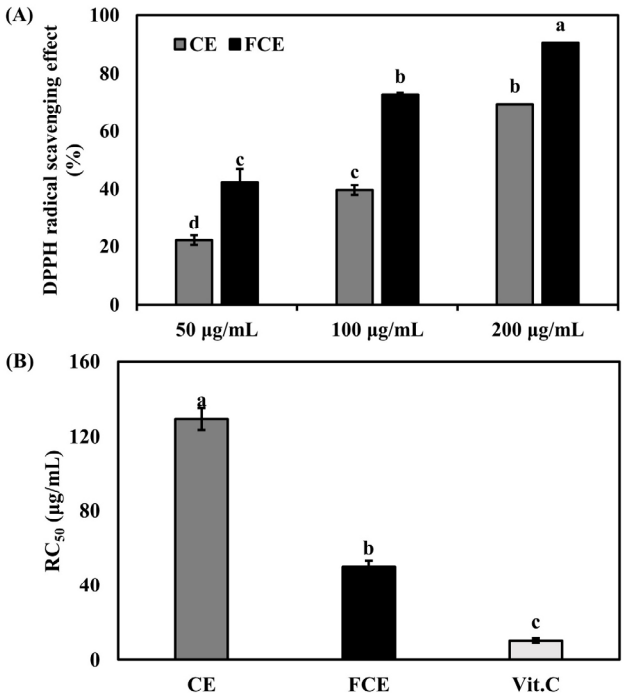

Fig. 4 DPPH radical scavenging activities of CE and FCE. (A) DPPH radical scavenging activity by concentration, (B) RC50: Amount required for 50% reduction of scavenging activity. Data are expressed as mean ± standard deviation (n = 3). The different letters indicate significant differences at $p < 0.05$ using Duncan's multiple range test

DPPH and ABTS radical scavenging activity of CE and FCE

DPPH radical scavenging activity was measured using the principle that DPPH radical, which is a kind of free radical and is known to cause oxidation in the body, is reduced by antioxidants (Fig. 4). As a result, the scavenging activity of 22.06%~69.49% was shown at the concentration of CE 50~200 μg/mL, and at the same concentration, the FCE showed a higher scavenging activity of 42.28%~90.44%. In particular, in the case of RC_{50} values, it was confirmed that CE and FCE inhibited DPPH radicals by 50% at concentrations of 129.44 ± 5.85 and 50.00 ± 3.47 μg/mL, respectively, and FCE was about 2.5 times lower than CE. The RC_{50} value of ascorbic acid as a control group was measured to be 10.24 ± 1.24 μg/mL.

ABTS radical scavenging activity was measured using the principle that blue-green ABTS radical cations formed by reaction with potassium persulfate are removed by antioxidants and decolorized (Fig. 5). Similar to DPPH radical scavenging activity, ABTS radical scavenging activity was measured to be 26.06%~69.50% at a concentration of 50~200 μg/mL CE. At the same concentration, FCE was 65.89% to 93.96%, confirming that it exhibited higher scavenging activity than CE.

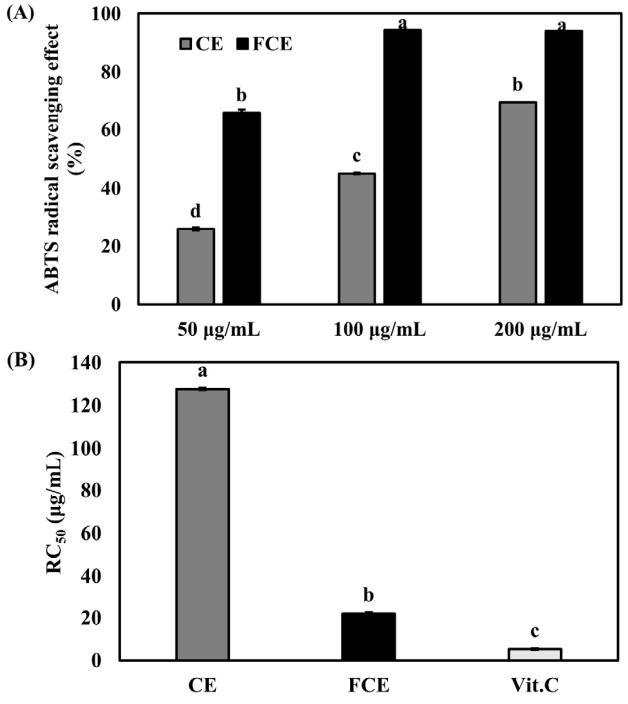

Fig. 5 ABTS radical scavenging activities of CE and FCE. (A) ABTS radical scavenging activity by concentration, (B) RC_{50}: Amount required for 50% reduction of scavenging activity. Data are expressed as mean ± standard deviation (n = 3). The different letters indicate significant differences at $p < 0.05$ using Duncan's multiple range test

FRAP activity of CE and FCE

The FRAP activity of CE and FCE is shown in Figure 6. The reducing power increased the activity in a concentration-dependent manner of the CE and FCE, and in particular, FCE showed significantly the highest FRAP activity at all concentrations.

Discussion

Phenolic compounds are one of the secondary metabolites commonly found in plants and occupy the largest portion, among which flavonoids are the largest class of polyphenols synthesized by plants and are known to have various physiologically active functions (Beecher 2003). In this study, as a result of measuring changes in the active ingredients of CE and FCE, it was confirmed that the total polyphenol (Fig. 2) content was increased by fermentation, and the pattern of the silymarin derivative compound (Fig. 3) was changed. It can be inferred that fermentation using lactic acid bacteria has a physical effect on the tissues of *Cirsium Lineare (Thunb.)*, so that the elution of physiologically active compounds is easy, and it is also

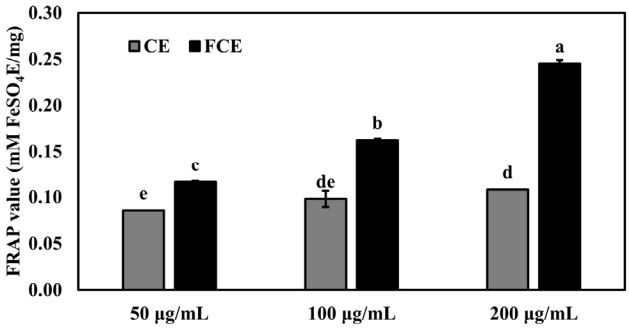

Fig. 6 Reducing power of CE and FCE. FRAP value was analyzed as $FeSO_4$ equivalent ($FeSO_4E$) mM/mg of extract. Data are expressed as mean ± standard deviation (n = 3). The different letters indicate significant differences at $p < 0.05$ using Duncan's multiple range test

expected that an increase or generation of useful physiologically active compounds can be expected through microbial metabolism.

The scavenging activity of DPPH and ABTS radical scavenging activity increases as the content of phenolic compound increases, and it is known that DPPH radical and ABTS scavenging activity have a significant correlation (Jeong et al. 2007). In addition, although most phenolic compounds effectively remove radicals, phenolic compounds that selectively act depending on the substrate of radicals exist. Therefore, in this study, both DPPH and ABTS radical scavenging activities were measured to evaluate antioxidant activity. FCE RC_{50} was 50.00 ± 3.47 μg/mL, which was about 2.5 times higher than that of CE RC_{50} (129.44 ± 5.85 μg/mL) (Fig. 4). This is considered to be because the FCE contains a large amount of phenols and flavonoid compounds, which are compounds with antioxidant activity. According to a previous study, polyphenolic compounds were reported to effectively remove DPPH radicals (Villaňo et al. 2007), and it was confirmed that the higher the polyphenolic content, the higher the DPPH radical scavenging activity. As a result of a study reporting a high correlation between polyphenolic compounds and ABTS radical scavenging activity (Choi et al. 2007), it was also confirmed that the higher the polyphenol content, the higher the ABTS radical scavenging activity. ABTS radical scavenging activity generally showed relatively higher activity than DPPH radical scavenging activity. Most phenolic compounds effectively remove radicals, but in the case of flavonoids, radical scavenging activity varies depending on the position of the substituent (Park and Kim 2004), It is thought that this is because, depending on the type of polyphenol, ABTS radical is removed but DPPH radical cannot be removed (Wang et al. 1998). Therefore, it is judged that FCE contains a lot of active

ingredients that can effectively remove DPPH and ABTS radicals. The content of the component was increased, which is thought to be the result of having more influence on the DPPH and ABTS radical scavenging effect.

FRAP is a method for measuring the degree of reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) colored by an antioxidant (Benzie and Strain 1996). The reducing principle of antioxidants is to suppress the chain reaction of free radicals by donating hydrogen atoms, thereby exhibiting antioxidant activity (Duh et al. 1999). When testing antioxidant activity, various methods are recommended to evaluate antioxidant efficacy because the measurement principle and reaction mechanism are different for each experiment and the degree of response to specific components is different (Patthamakanokporn et al. 2008). Therefore, in this study, as a result of measuring the FRAP activity of CE and FCE, it was confirmed that the reducing ability of FCE was superior to that of CE, similar to the radical scavenging ability.

In this study, changes in polyphenol content and silymarin derivative pattern, DPPH, ABTS radical scavenging activity, and FRAP activity were confirmed using CE and FCE to investigate the effects of *Cirsium Lineare (Thunb.)* on antioxidant components and effects by fermentation of lactic acid bacteria. As a result, it was confirmed that the polyphenol content of *Cirsium Lineare (Thunb.)* seeds was increased by fermentation of lactic acid bacteria, and there was also a pattern change in silymarin derivative pattern. In particular, it was confirmed that FCE increased DPPH, ABTS radical scavenging activity and FRAP activity compared to CE, similar to the content change. When CE and FCE were compared, FCE showed a better effect overall, which is believed to be due to the increase in the active ingredient of *Cirsium Lineare (Thunb.)* through the fermentation process. In conclusion, this study suggested the possibility that FCE prepared through lactic acid bacteria fermentation could be utilized as a powerful antioxidant material.

Conflict of Interest

The authors declare that they have no conflict of interest.

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