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Anticancer effects of washed-dehydrated solar salt doenjang and its metabolites

So-Young Lee[1,2], Geun-Hye Hong[1,2] and Kun-Young Park[1,2*]

Abstract

In this study, the anticancer effect of doenjang according to the type of salt was investigated. Three samples were prepared: doenjang made with purified salt, doenjang made with generally manufactured solar salt, and doenjang made with washed and dehydrated solar salt (WDSD). In mice in which colon cancer was induced with azoxymethane/dextran sodium sulfate, doenjang made with solar salt, especially doenjang made with washed and dehydrated solar salt, was found to have a much higher colon cancer inhibition effect. WDSD significantly promoted the mRNA expression of apoptosis-related factors such as Bcl-2–associated X protein (Bax) and caspase 9 and the cell cycle arrest-related factors p53 and p21, and conversely significantly reduced the mRNA expression of apoptosis inhibitors such as B-cell lymphoma-2 (Bcl-2) ($p < 0.05$). Additionally, metabolites were investigated to determine which substances in WDSD exhibit this anticancer effect. As a result, the contents of isoflavone and soyasaponin B in the form of aglycons such as genistein, daidzein, and glycitein, which are known to have anticancer and anti-inflammatory properties, were found to be significantly high. Therefore, the results confirmed that doenjang prepared with washed and dehydrated solar salt has superior anticancer potential against colon cancer, and that various active ingredients contribute to the improvement of this functionality.

Keywords Anticancer, Washed-dehydrated solar salt, Doenjang, Metabolite

Introduction

Doenjang is a traditional Korean food with a long history. The origin of soybeans, which are the main ingredients of doenjang, is believed to be the Korean Peninsula and Manchuria [1], and it is presumed that soybeans began to be cultivated and made into jang during the Goguryeo period [2]. It is not known exactly when the history of doenjang in Korea began, but historical records resembling doenjang predate the Silla Dynasty, indicating that the form of a jang existed from 1200 years ago [3]. However, considering the origin of soybeans, it is estimated that they have a history of 2000 years [4]. Old records such as Dongyijeon record that people during the Goguryeo period had already developed a variety of fermented foods [2], and in *Haedongyeoksa*, a history book of the Balhae Empire, it is recorded that jang are a specialty of Balhae [5]. In addition, the *Samgukyusa* contains a record that jang was used not only for food but also to treat wounds [5], and the *Samguksagi* records that jang was included as an item of wedding gifts for King Sinmun [6]. In addition, various historical books contain records of fermented foods such as doenjang and ganjang. During the mid-Joseon dynasty, the document *Jungbosanlimkyungji* was compiled, detailing methods for making meju using soybeans, as well as the selection criteria for water and salt, establishing foundational techniques for contemporary doenjang production [7].

Salt is an essential material when making doenjang [8], and plays an important role in suppressing the growth of spoilage microorganisms during the fermentation period and helping salt-tolerant fermentation microorganisms

*Correspondence:
Kun-Young Park
kypark9004@gmail.com
[1] Department of Food Science and Biotechnology, CHA University, Seongnam, Gyeonggi-do 13488, South Korea
[2] IMMUNOBIOTECH Corp., Seoul 06628, South Korea

grow easily [9]. Additionally, the minerals in salt serve as a source of nutrients for the growth of microorganisms during the fermentation period of fermented foods such as kimchi, doenjang, and ganjang [10]. These mineral sources vary depending on the type of salt, which appears to be due to differences in manufacturing methods [11]. For example, purified salt is 99% high purity sodium chloride, but solar salt contains various minerals such as iron (Fe), magnesium (Mg), calcium (Ca), and potassium (K) [12].

According to a previous study, when ganjang was manufactured according to the type of salt, ganjang made with solar salt, especially solar salt with the bittern removed, showed excellent quality [13]. It was also reported that it showed a significant anti-cancer effect compared to other groups such as purified salt, etc. [13]. A study by Yu et al. [14] also reported that making kimchi with washed and dehydrated sea salt improves anti cancer [14] functionality. Thus, using solar salt, which is rich in minerals, when manufacturing fermented foods is known to affect the growth and fermentation process of microorganisms [15, 16], and improve taste and functionality [17].

Therefore, in this study, we aimed to investigate the anticancer functionalities against colorectal cancer-induced mice and metabolic differences in fermented doenjang based on salt types, specifically purified salt (PS), generally manufactured solar salt (GS), and washed-dehydrated solar salt (WDS). The conditions related to meju, water, and fermentation were kept constant, varying only the type of salt used.

Materials and methods

Sample preparation

The meju used in this study was a grain-type meju purchased from Alali Food Co. (Goryeong, Korea), which was manufactured by inoculating locally grown soybeans (*Glycine max*.) with *Aspergillus oryzae* (0.02% w/w) as the starter [18]. Three types of salt were used: purified salt (PS), generally manufactured solar salt (GS), and washed-dehydrated solar salt (WDS). PS was used a product, which is currently on the market, while the two types of solar salt (GS, WDS) were provided from Taepyeong salt (Shinan, Korea).

Doenjang was fermented at 34 °C for a duration of 4 months, following the mixture of meju, salt, and water in a ratio of 1:1:4, as described in previously reported paper [19]. After fermentation was completed, doenjang (meju, solid part) and ganjang (liquid part) were separated, and PS doenjang (PSD), GS doenjang (GSD), and WDS doenjang (WDSD) were used in the experiment.

In vivo anticancer effects of doenjang

The mice used in the experiment were C57BL/6 male mice (6-weeks old) purchased from Orient Bio (Sungnam, Korea) and were bred at a temperature of 22 ± 2 °C, relative humidity of 55 ± 2%, and a light/dark cycle for 12 h [20]. After a one-week acclimation period, mice were categorized into (1) normal (Nor), (2) control (Con), (3) PSD, (4) GSD, and (5) WDSD groups. Except for the Nor group, the other groups were administered 10 mg/kg of azoxymethane (AOM, Sigma-Aldrich Co., St. Louis, MO, USA) intraperitoneally to induce colon cancer, and one week of 2% dextran sodium sulfate (DSS, Reagent grade, M.W. 36,000–50,000; MP Biomedicals, LLC, Illkrich, France) was provided in drinking water at 2 and 5 weeks after AOM administration [19].

The mouse diet was fed AIN-93G, and each sample group was prepared by adding 10% of the weight of the feed and allowed to freely consume diet and water. The experiment was conducted for 8 weeks, and the anticancer effect was experimented using mouse colon tissue that was sacrificed after that. This experiment was conducted with permission from the Animal Ethics Committee of CHA University (IACUC200004).

RT-qPCR analysis

RNA was isolated from colon tissue of each group using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA), and a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA) was used to quantify the isolated RNA. Afterwards, the quantified RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Co.), synthesized into cDNA, and used in experiments [21]. This cDNA was mixed with related primers (Bioneer, Daejeon, Korea) and SYBR green (Solis BioDyne, Tartu, Estonia), and gene expression was analyzed using a thermal cycler Bio-Rad CFX-96 real time system (Bio-Rad, Hercules, CA, USA) [22]. The expression levels of genes including Bcl-2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), caspase 9, p53, and p21 were measured in the colon tissues of mice from each group.

The 18S rRNA gene was employed as the housekeeping gene for normalization. The sequence of each gene is 18S, forward 5′-CAG CCA CCC GAG ATT GAG CA-3′, and reverse: 5′-TAG TAG CGA CGG GCG GTG TG-3′; Bax, forward 5′-TGC TTC AGG GTT TCA TCC AG-3′, and reverse: 5′-GGC GGC AAT CAT CCT CTG-3′; Bcl-2, forward 5′-CAG CTG CAC CTG ACG CCC TT-3′, and reverse 5′-GCC TCC GTT ATC CTG GAT CC-3′; caspase 9, forward 5′-CTA GTT TGC CCA CAC CCA GT-3′, and reverse 5 ′-CTG CTC AAA GAT GTC GTC CA–3′; p53,

forward 5′-ATG GAG GAG CCG CAG TCA GA-3′, and reverse 5′-TGC AGG GGC CGC CGG TGT AG-3′; p21, forward 5′-ATG TCA GAA CCG GCT GGG G-3′, and reverse 5′-GCC GGG GCC CCG TGG GA-3′ [22]

Metabolomic analysis

Metabolites from soybean paste were extracted with 80% methanol, and terfenadine (Sigma-Aldrich, Missouri, USA) was used as an internal standard. Metabolite analysis is almost identical to the Gu et al. [23] method.

Doenjang extract was analyzed using ultra performance liquid chromatography-quadrupole-time of flight (UPLC-Q-TOF) mass spectrometry (MS) (Xevo G2-S, Waters) equipped with an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 um; Waters). The mobile phase for the extracted sample was distilled water containing 0.1% formic acid and acetonitrile (ACN) containing 0.1% formic acid. The flow rate was 0.35 mL/min, the analysis time was 9 min, and the column temperature was 40 °C. Metabolites that passed through the column were analyzed in Q-TOF MS-positive mode. The capillary and sampling cone voltages were set to 3 kV and 30 V, respectively, the source and desolvation temperatures were set to 100 °C and 400 °C, and the desolvation flow rate was set to 800 L/h. MS data were collected in the m/z 50–1500 range with a scan time of 0.2 s. To ensure accuracy and reproducibility in all analyses, leucine-enkephalin ([M + H] = 556.2771 Da) was used as a lock mass and analyzed at a frequency of 10 s. In addition, quality control by mixing all samples was analyzed before and after analysis and once per five samples. MS/MS spectra of metabolites were collected at 10–30 eV, and m/z 50–1500.

Statistical analysis

The results of the RT-qPCR experiment are expressed as mean ± standard deviation (SD), and significance was tested by performing one-way analysis of variance (ANOVA) and Duncan's multiple range test at the $p < 0.05$ level. All statistical analyses were performed using SPSS (ver. 18.0, SPSS Inc., Chicago, IL, USA) statistical program.

The doenjang metabolite analysis data were subjected to multivariate statistical analysis using SIMCA-P + version 15.0.2 (Umetrics, Umeå, Sweden), and partial least squares-discriminant analysis (PLS-DA) was used to visualize the analysis results. To evaluate the PLS-DA used, R2X, R2Y, Q2, and p-values were evaluated, and cross-validation was performed using a permutation test. In addition, the relative abundances of metabolites were statistically analyzed using Duncan's test ($p < 0.05$) using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Effects of WDSD on mRNA expression of the apoptosis-related genes

Apoptosis is an essential element of various processes that occur in multicellular organisms [24] and is generally regarded as programmed cell death [25]. It is influenced by several regulators, and defects in apoptosis regulation cause various diseases such as cancer [26]. The Bcl-2 family is involved in directly inducing or suppressing apoptosis [25]. Among these, Bax is a pro-apoptotic gene that activates apoptosis [27], but Bcl-2 is an anti-apoptotic factor that suppresses apoptosis [28]. Therefore, if Bax is activated and Bcl-2 is inhibited, it can cause inhibition of cancer cell growth through apoptosis [29].

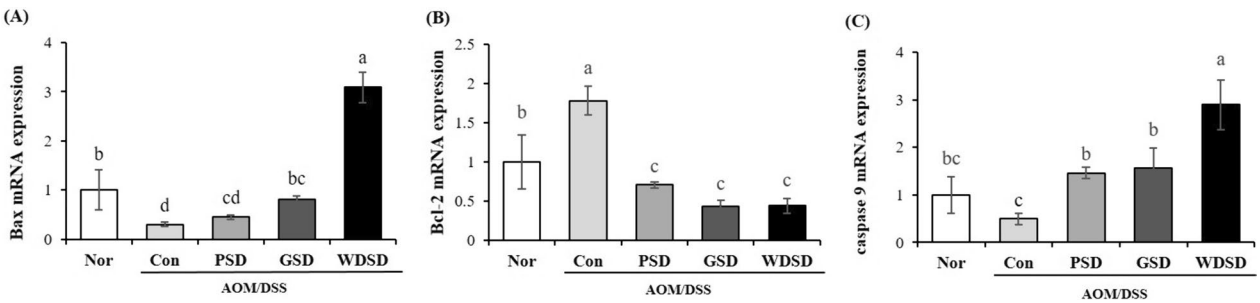

Fig. 1 Effects of doenjang on the mRNA expression of Bax (**A**), Bcl-2 (**B**) and caspase 9 (**C**) genes in the colon of C57BL/6 mice with AOM/DSS induced colitis associated colon cancer. Nor: AIN-93G, Con: AOM/DSS + AIN-93G, PSD: AOM/DSS + 10% purified salt doenjang with AIN-93G, GSD: AOM/DSS + 10% generally manufactured solar salt doenjang with AIN-93G, WDSD: AOM/DSS + 10% washed-dehydrated solar salt doenjang with AIN-93G. [a-d]Means with different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test. AOM, azoxymethane; DSS, dextran sodium sulfate; PSD, purified salt doenjang; GSD, generally manufactured solar salt doenjang; WDSD, washed-dehydrated solar salt doenjang

The results of examining the anticancer effects of fermented doenjang based on different types of salt (Fig. 1) revealed significantly higher mRNA expression of Bax in the doenjang consumption group than in the control group (Fig. 1A). Particularly, even for the same type of doenjang, those prepared with washed-dehydrated solar salt exhibited a 10.1 fold increase compared to the control group, and it was significantly 3.8 and 6.8 fold higher than the doenjang prepared with purified salt and generally manufactured solar salt, respectively ($p < 0.05$).

On the other hand, the mRNA expression of Bcl-2 (Fig. 1B) was significantly higher in the Con group but lower in the doenjang consumption group ($p < 0.05$), and was approximately 1.6 times lower in doenjang prepared with solar salts (GSD, WDSD) than in those prepared with purified salt.

According to the study by Lee and Chang [30], treatment with doenjang extracts made with solar salt significantly inhibited the growth of AGS (gastric cancer cells) and HT-29 (colorectal cancer cells) cells compared to those treated with purified salt doenjang, and a higher level of apoptosis induction was observed in the solar salt doenjang. A study by Lee et al. also reported that washed-dehydrated solar salt ganjang exhibited anticancer functionality by controlling the expression of apoptosis-related factors such as Bax and Bcl-2 [13]. In particular, Bax is known to be associated with the suppression of colon tumors occurrence [31]. In a previous study by Lee et al. [19], a pathological analysis of colon tissue was performed and results showed that the colon tissue of the WDSD group with high Bax expression recovered similar to Nor compared to other groups and it was confirmed that colonic health had improved [19].

Caspase 9 is also an apoptotic caspase [32] and is a factor that induces apoptosis under the influence of activated Bax [33]. The expression rate of caspase 9 was high in the group that consumed doenjang (Fig. 1C), which was 5.8 times higher in the WDSD group than in the Con group and 1.8 times higher in the WDSD group than in the GSD group ($p < 0.05$). Therefore, washed-dehydrated solar salt doenjang is thought to have an inhibitory effect on colon cancer by suppressing the expression of Bcl-2, activating Bax, and further activating apoptosis by enhancing the expression of caspase 9.

Effects of WDSD on mRNA expression of the cell cycle arrest-related genes

P53 is an important tumor suppressor gene that regulates various cellular responses to prevent cancer development, including DNA repair, cell cycle arrest, cell senescence, cell differentiation, and death [34]. In addition, p53 is also related to the expression of p21 [35], and p21 is also an important regulator associated with the cell cycle that inhibits cell proliferation, and is known to inhibit the cell growth of malignant tumors [36].

The mRNA expression of p53 was higher in the groups that consumed doenjang than in the Con group (Fig. 2A), and was higher in GSD and WDSD made with two types of solar salt than in PSD. In particular, WDSD was expressed 7.1-fold higher than Con, 3.7-fold higher than PSD, and 1.8-fold higher than GSD manufactured with general solar salt ($p < 0.05$). The expression of p21 mRNA displayed a similar trend (Fig. 2B), with the Con group exhibiting significantly lower expression than the other groups ($p < 0.05$). Additionally, in comparison to PSD, GSD showed a 1.3-fold higher expression, whereas it was 2.7-fold lower compared to WDSD, indicating functional differences based on the type of solar salt used.

This is consistent with research results showing that intake of WDSD effectively suppressed colon cancer by regulating the protein expression of various factors related to cancer cell development and growth, such as

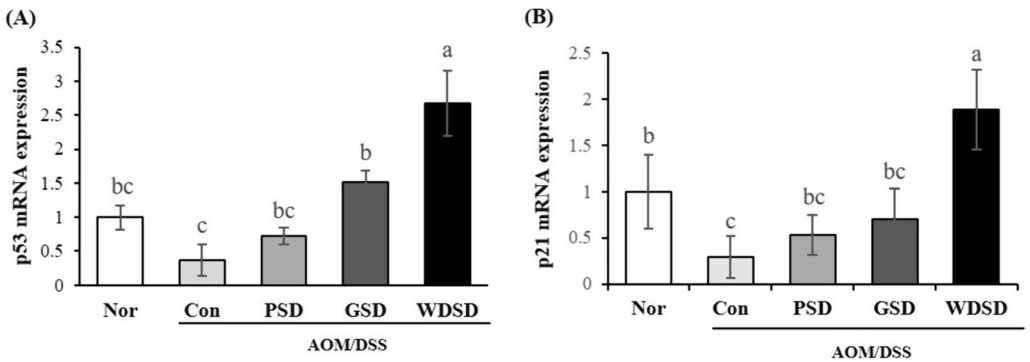

Fig. 2 Effects of doenjang on the mRNA expression of the p53 (**A**) and p21 (**B**) genes in the colon of C57BL/6 mice with AOM/DSS induced colitis associated colon cancer. Nor: AIN-93G, Con: AOM/DSS + AIN-93G, PSD: AOM/DSS + 10% purified salt doenjang with AIN-93G, GSD: AOM/DSS + 10% generally manufactured solar salt doenjang with AIN-93G, WDSD: AOM/DSS + 10% washed-dehydrated solar salt doenjang with AIN-93G. $^{a\text{-}c}$Means with different letters on the bars are significantly different ($p < 0.05$) by Duncan's multiple range test

apoptosis and cell cycle arrest [19]. Yu et al. [14] and Park et al. [37] reported that the anticancer and antiobesity functionality of kimchi manufactured with washed and dehydrated sea salt was improved, because bittern was removed through washing and dehydration during the manufacturing process of sea salt, and minerals such as Mg and S were removed. Our experimental results likewise suggest that the anticancer effect observed in doenjang made with washed-dehydrated solar salt may be attributed to the same factors. Therefore, it is believed that doenjang with enhanced colon cancer prevention effects can be produced by using solar salt from which bittern water has been removed through washing and dehydration processes.

Metabolite analysis using UPLC-Q-TOF MS

It is known that these various functions (such as anticancer effects) of doenjang are due to metabolites produced during fermentation [38]. Therefore, we determined what metabolites are produced when doenjang is manufactured with washed-dehydrated solar salt (WDSD), and what differences exist between doenjang manufactured with other types of salt (especially purified salt), and metabolite profiling was performed using UPLC-Q-TOF-MS.

The results of confirming the total ion chromatogram for the PSD and WDSD methanol extracts through LC/MS are shown in Fig. 3. Mass spectrometry data processing, including m/z, retention time, and ion intensity, was performed using UNIFI version 1.8.2 (Waters), and metabolites were analyzed based on online databases including the ChemSpider database, METLIN database, and human metabolome database. Thus, 13 metabolites were identified: pyrazine; tryptophan; Asp-Phe; dimethylquinoline; genistein- related compounds; daidzein; glycitein; genistein; B soyasaponin Bb; lysophosphatidylcholine (LPC) (18:2); parinaric acid; ganoderol B; and monolinolenin. The mass spectrum data of peaks 1–13 of Fig. 3 are shown in Table 1. In particular, except for tryptophan, the remaining metabolites had VIP values of over 1.0 [39], which showed that they significantly promoted the difference between the two groups. As a result of visualization using multivariate statistical analysis to confirm the differences between groups (Fig. 4), the quality parameter analysis of the PLS-DA model used in the comparative analysis showed high fit (R_2X: 0.563, R_2Y: 0.999) and prediction quality (Q_2, 0.969), and was confirmed as the p value was 8.35×10^{-4} (Fig. 4A). In addition, as a result of cross-validation through a permutation test (when $R2<1.0$ and $Q2<0.5$) (Fig. 4B), the PLS-DA model was confirmed to be statistically significant, and PSD and WDSD were confirmed to be clearly separated from each other on the PLS-DA score plot.

Boxplots was used to visualize the differences in metabolites for PSD and WDSD extracts, and the data were converted to Z values and displayed as heatmaps. The boxplot results (Fig. 5)-, show that among the 11 metabolites, WDSD showed a relatively higher content than PSD in the remaining metabolites except pyrazine, Asp-Phe, dimethylquinoline, and monolinolenin. In the heatmap results, the difference in metabolite content between the PSD and WDSD groups was clearly revealed (Fig. 6).

Genistein, daidzein, and glycitein are among the isoflavone that are functional components of soybeans, the main ingredient in doenjang [40]. They are easily absorbed into the body and are known to exhibit various physiological activities due to their high absorption rate in the intestines [41, 42]. In the case of genistein, anti-inflammatory effects were confirmed by reducing the frequency of rectal bleeding and diarrhea in mice with DSS induced colitis and significantly suppressing the expression of inflammation-related genes such as IL-1β and IFN-γ. [43], and it has been reported to have anticancer effects on HT-29 cells [44]. In addition, daidzein

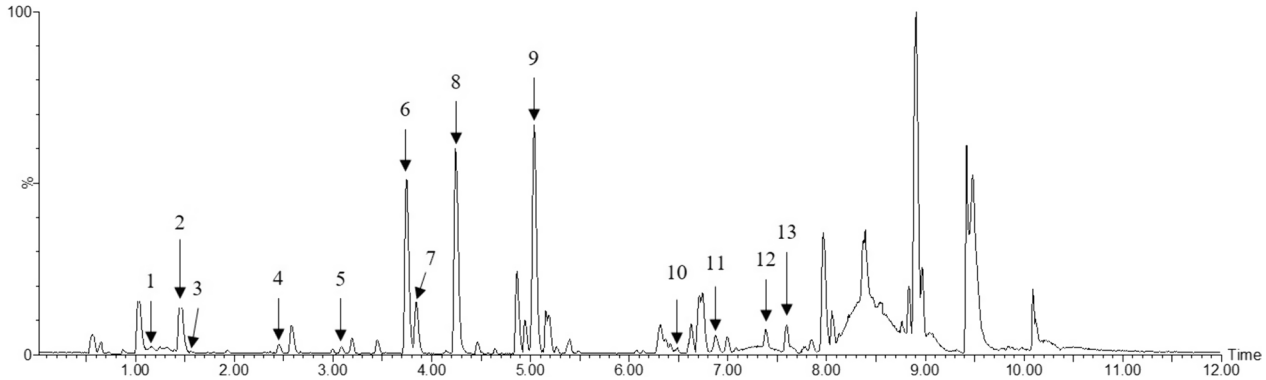

Fig. 3 Total ion chromatogram of PSD and WDSD methanol extracts. The peak numbers assigned on the peaks correspond to those in Table 1

Table 1 Identification of major metabolites contributing the separation among sample groups

No	RT[a] (min)	Compound	Exact mass[b] (M + H)	MS fragments	VIP[c]	*p* value[d]
1	1.17	Pyrazine	243.1324	197, 217	1.47	2.76E−07
2	1.46	Tryptophan	205.0949	188	0.19	7.32E−01
3	1.56	Asp-Phe	281.1122	235, 166, 120	1.41	5.00E−05
4	2.45	Dimethylquinoline	158.0932	143, 144, 130	1.37	2.19E−04
5	3.08	Genistein derived materials	271.0589	215, 153	1.17	7.09E−03
6	3.74	Daidzein	255.0633	199, 277, 237	1.06	2.24E−02
7	3.84	Glycitein	285.0738	270, 242, 197	1.01	3.20E−02
8	4.24	Genistein	271.0585	243, 215, 153	1.15	9.19E−03
9	5.04	B soyasaponin Bb	943.5280	797, 599, 441, 423	1.07	2.03E−02
10	6.49	LPC(18:2)	520.3398	184, 104	1.13	1.10E−02
11	6.89	Parinaric acid	277.2139	261, 121, 107	1.14	9.95E−03
12	7.38	Ganoderol B	441.3709	423, 405	1.43	1.05E−05
13	7.59	Monolinolenin	353.2665	261, 149	1.45	3.01E−06

[a] RT is retention time

[b] Exact mass was the mass calculated from a molecular formula using known masses of specific isotopes with the appropriate number of decimal places

[c] VIP is variable importance in the projection

[d] *p* values were analyzed by ANOVA with Duncan's multiple range test

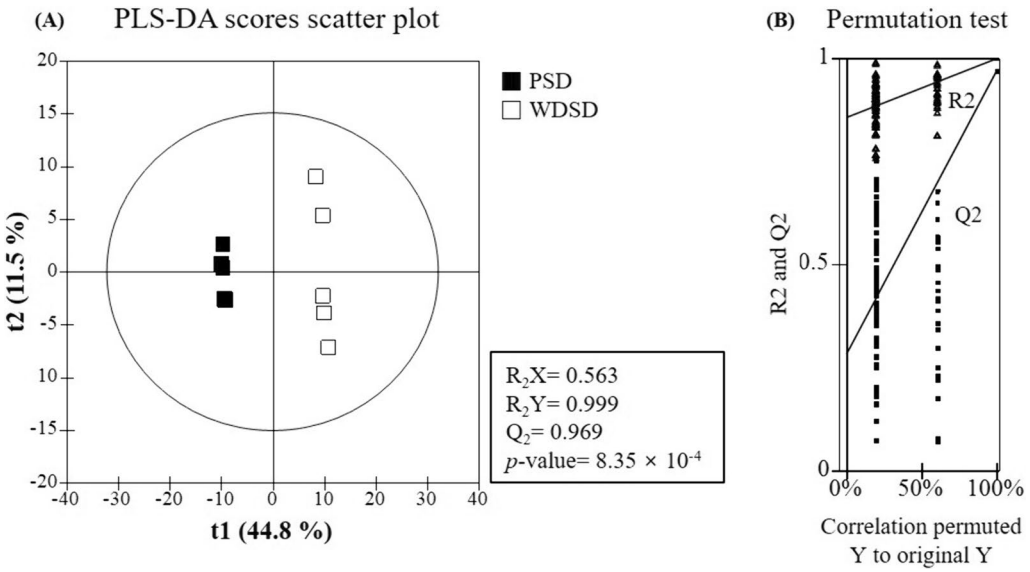

Fig. 4 Partial least squares discriminant analysis (PLS-DA) score plots of doenjang metabolites (**A**) and its quality parameters (**B**). Metabolites were analyzed using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS). The statistical acceptability of the PLS-DA model (**A**) was evaluated by R_2X, R_2Y, Q_2, and *p*-value and validated by cross validation with a permutation test (**B**)

has been shown to improve insulin resistance, plasma lipid changes, inflammation, etc., and effectively prevent type 2 diabetes [45]. In addition, isoflavones (genestein, dadizein, glycitein) are known to have antiobesity [46], antihypertension [47], anti-inflammatory [48], and antiallergic effects [49].

Soyasaponin is identified in legumes and is classified into forms A and B depending on its structure [50]. Soyasaponin B is related to most of the physiological activities of soybeans [50], and it has been found to have several health benefits such as liver damage protection [51], anticancer [52], and anti-inflammatory effects [53].

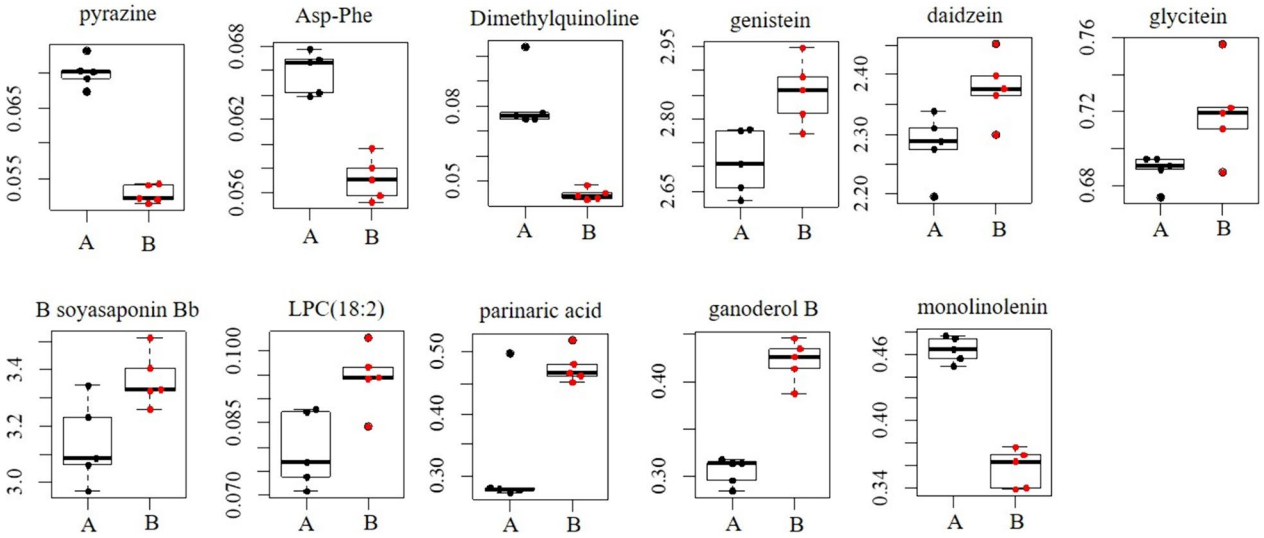

Fig. 5 Box plot analysis of PSD (**A**) and WDSD (**B**) metabolites

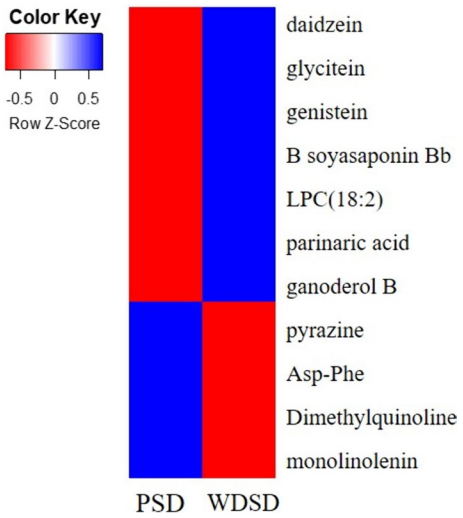

Fig. 6 Heatmaps of PSD and WDSD methanol extracts metabolites analyzed using UPLC-Q-TOF MS analysis

Ganoderol B is a ganoderol alcohol and is known to have a strong hypoglycemic effect [54] and an antiproliferative effect on human prostate cancer LNCaP cells [55].

These bioactive substances are mainly metabolites identified in doenjang made with washed and dehydrated sea salt rather than PSD, depending on the type of salt, even though doenjang has the same storage period and manufacturing method. The abundance of minerals in solar salt is presumed to enhance microbial growth and activity [12], leading to an increase in bioactive components in washed-dehydrated solar salt based doenjang. Moreover, in addition to the individual effects of these compounds, it appears that various compounds have a synergistic effect when combined, showing improved anticancer effects in WDSD.

In summary, this study confirmed the inhibitory effect on colon cancer through the in vivo anticancer activity of doenjang prepared using washed-dehydrated solar salt. The results revealed that effective suppression of cancer cell initiation and growth could be achieved by regulating the expression of various factors related to cell apoptosis and cell cycle arrest, such as Bax, caspase 9, p53, and p21. In addition, metabolites with mainly anticancer, antioxidant, and anti-inflammatory effects were detected in WDSD, which is believed to have an effect on improving the functionality.

Therefore, appropriately regulated mineral content through the washing and dehydration process influences microbial metabolism, counteracting the negative impact of salt during the fermentation process and contributing to the production of various bioactive compounds in doenjang. These findings are considered a form of the Korean paradox [56].

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Author contributions
So-Young Lee: Conceptualization, methodology, validation, formal analysis, data curation, writing—original draft. Geun-Hye Hong: Validation, data curation, visualization. Kun-Young Park: Conceptualization, writing—review and editing, resources, supervision, project administration.

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Availability of data and materials
The data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
This study was approved by the Animal Ethics Committee of CHA University (Approval No. IACUC200004).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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