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Toxicological evaluation of lotus, ginkgo, and garlic tailored fermented Korean soybean paste (*Doenjang*) for biogenic amines, aflatoxins, and microbial hazards

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ABSTRACT

The present study aimed to develop a consortium of nutritive fermented food products, supplemented with phytochemicals, with reduced toxicological contents. We developed new flavored *Doenjang* products (protein rich) fermented with lotus, ginkgo, and garlic plant extract-based *Meju* (termed as EMD) as the starter culture and by using traditional *Meju* (termed as TMD), where these plant extracts were added later during the fermentation process. Fermented *Doenjang* samples were analyzed for reduced levels of biogenic amines (BAs), aflatoxins, and microbial hazards, (including *Bacillus cereus)* as well as for their nutritive contents and antioxidant potential, after varying periods of fermentation (0, 3, 6, 9 and 12 months). All *Doenjang* samples prepared using plant extracts and their mixtures (1% and 10%) showed desired reduction in *B. cereus* counts, BAs, aflatoxins, and other foodborne pathogens as well as showed potent antioxidant abilities, including phenolic/flavonoid contents. Based on the higher efficiency in reducing various toxicants, *Ginkgo biloba* leaf extract added TMD samples were selected for the development of *Doenjang* products as an innovative approach, with great potential to improve the quality and safety of soybean fermented products in the Korean market, offering enhanced health benefits and reduced risks of toxicity.

1. Introduction

Food products are manufactured and distributed worldwide; however, drawbacks in regulatory guidelines have led to an increased risk to the consumer's health. Heavy metals, pesticides, pharmaceuticals, personal care products, food toxins such as biogenic amines (BAs), and aflatoxins have been detected in different food matrices, worldwide (Papageorgiou et al., 2018). BAs, aflatoxins, and microbial foodborne hazards are of major concerns in fermented food products, which cause life threatening and toxicological reactions in the human body. Presence of high levels of BAs and aflatoxins in fermented foods may be attributed to poor hygiene standards, inferior quality of raw materials, poor manufacturing practices, contamination by specific bacteria, ripening period, and the type of starter culture used (Ekici and Omer, 2018).

Common intoxication symptoms of BAs (histamine, tyramine, tryptamine, and β-phenylethylamine) include nausea, respiratory distress, hot flushes, perspiration, heart palpitations, headache, rashes, oral burning, and hypo- and hypertension. Biogenic polyamines, such as putrescine, cadaverine, spermidine, and spermine, also enhance the toxicity of BAs via interference with detoxification mechanisms (Jeon et al., 2018). The presence of hazardous levels of BAs is associated with a relevant growth (> 7 log cfu/g) of decarboxylating microorganisms. Therefore, researchers have proposed microbial quality indices based on food BA content to indicate excessive microbial contamination and proliferation (Al Bulushi et al., 2009). Due to high levels of BAs reported in soybean-based fermented food products (Jang et al., 2006; Lee et al., 2010), researchers are keen to develop products with low

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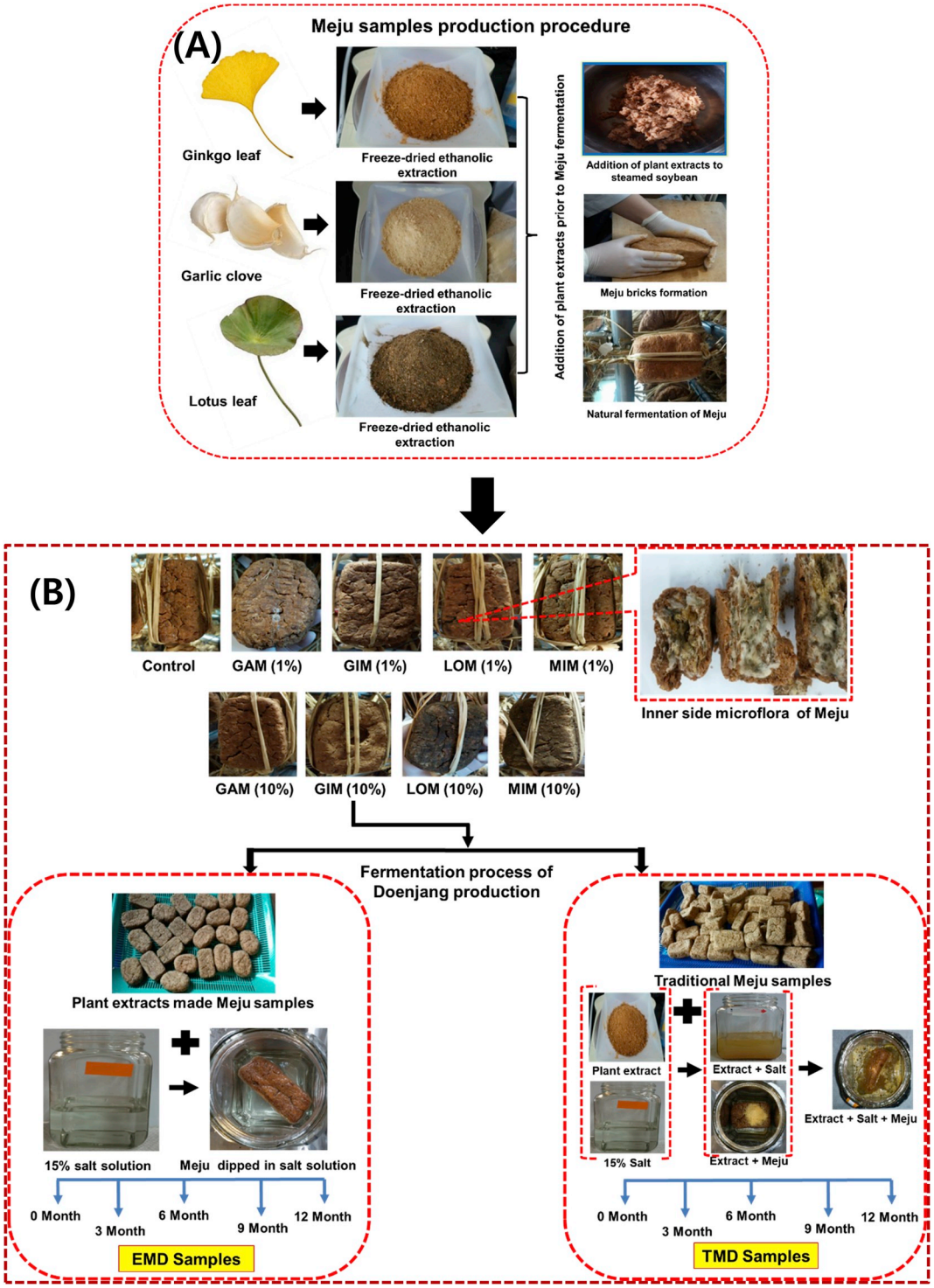


Fig. 1. Scheme for the development of *Doenjang* products with a combination of lotus, ginkgo, and garlic plant extracts (A) production of plant extracts added *Meju*; (B) production of EMD and TMD *Doenjang* products.

risks of toxicity.

Aflatoxins are secondary metabolites produced by mycotoxins that exist in food and agricultural products, especially in soybean, peanut, paddy rice, wheat, and corn (Jia et al., 2019). *Aspergillus* species, specifically *Aspergillus flavus* and *Aspergillus parasiticus*, are the dominant producers of aflatoxins in fermented food and feed products (Kademi et al., 2017). Decontamination of food contaminated by mycotoxins is a tedious process or even impossible to achieve. This is due to the resistance of many known mycotoxins to extreme environmental conditions, as well as to physical and biological treatments, specifically designed for their inactivation/detoxification (Paterson and Lima, 2010). Therefore, when they accumulate in humans or animals, they exert toxicological effects.

Conventional strategies for prevention of mycotoxin poisoning often require both pre- and post-harvest approaches. Pre-harvest approaches deal with controlling the fungal contamination in the field, while post-harvest approaches deal with sorting and proper storage. Often, these approaches are not sufficient, requiring additional processing for

decontamination and detoxification of the food and feed products. Mycotoxins are very heat-stable and are difficult to eliminate by conventional thermal operations. Among these strategies, typical methods that have been reported include breeding for aflatoxin resistant crop varieties, prevention of aflatoxin producing fungal contamination, inhibition of aflatoxin production, removal of aflatoxin by physical methods, inactivation by chemical agents, and biological detoxification by microorganisms or plant products and their metabolites.

Doenjang is a protein-rich traditional food product and flavoring ingredient of Korea produced by the fermentation of soybeans. Traditionally, in the preparation of starter culture (*Meju*) for *Doenjang*, soybeans are fermented by *Bacillus* species in the early stage, followed by the secondary microflora of *Aspergillus* species, such as *A. oryzae* and *A. niger* (Kwon et al., 1998). The processing methods of traditional *Doenjang* differ depending on its region of origin. Therefore, a standardized fermentation process and protocol are needed for preparation of the starter culture to ensure a safe production of *Doenjang,* with improved quality and reduced toxicological risk.

Traditionally, in Korea, fermented soybean paste (*Doenjang*) is preferentially prepared with natural microflora (*Bacillus*, *Aspergillus*, *Penicillium*, and *Rhizopus* spp.); however, during fermentation the products may get contaminated and form hazardous toxicants. In our earlier study, we detected toxicants (BAs and aflatoxins) in *Doenjang* samples above the standard safety limit defined by the food regulatory agencies (Kim and Kim, 2012; Shukla et al., 2010). Subsequently, we produced microbial starter cultures for *Doenjang* production and measured the BA and aflatoxin contents (Shukla et al., 2014); we found that microbial cultures from *Doenjang* samples had minimal amounts of BAs and aflatoxins (Shukla et al., 2014).

In the previous study, we used *Doenjang* starter culture called *Meju*, supplemented with plant extracts (lotus, ginkgo, and garlic) for a short term fermentation period (1 month) and monitored the levels of aflatoxins, BAs, and microbial hazards. However, the possibility of contamination with toxicants during the long term *Doenjang* fermentation (12 months) could not be eliminated. Therefore, the present study was extended for monitoring the reduction of toxicants in *Doenjang* during fermentation for different time periods using *Meju* samples with added plant extracts as the starter cultures. To the best of our knowledge, there are no detailed studies on the fermentation of *Doenjang* samples employing *Meju* starter, fortified with lotus, ginkgo, and garlic extracts, or on evaluation of their safety and nutritional qualities. Therefore, in this study, we aimed to perform fermentation of *Doenjang* samples using selected plant extracts and *Meju* starters to achieve better nutrition quality of *Doenjang* samples with less toxic contaminants.

2. Material and methods

2.1. Chemicals and reagents

All tested biogenic amines (BAs) and solvents used were of high analytical and chromatographic grade, respectively, with ultrafine purity, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The veratox aflatoxin test kit was obtained from Neogen Corp (Lansing, MI, USA).

2.2. Production of Doenjang samples fortified with lotus, ginkgo, and garlic plant extracts

In the present study, previously produced *Meju* starter cultures supplemented with plant extracts of lotus, ginkgo, and garlic (1% and 10%), and their mixture (1:1:1 ratio) were used for the production of *Doenjang* fermented samples. The preparation procedure of making *Meju* with plant extracts is summarized in Fig. 1A. *Doenjang* samples were collected after 0, 3, 6, 9, and 12 months of fermentation period. Additionally, *Doenjang* samples with added plant extracts were prepared in two ways in order to evaluate the appropriate stage for the addition of the plant extracts; viz., (i) *Doenjang* produced using *Meju* prepared with plant extracts [*Doenjang* with extract *Meju* (EMD)] and (ii) *Doenjang* produced using traditional *Meju*, and plant extracts added during the course of fermentation at the same concentration and the ratio [*Doenjang* with traditional *Meju* (TMD)]. A detailed production scheme of *Doenjang* is shown in Fig. 1B.

2.3. Analysis for determining biogenic amine (BAs) content

2.3.1. Standard amines and other reagents

Stock solutions of all nine BAs, such as agmatine sulfate (AGM), tryptamine hydrochloride (TRP), 2-phenylethylamine (PHE), putrescine dihydrochloride (PUT), cadaverine dihydrochloride (CAD), histamine dihydrochloride (HIS), tyramine hydrochloride (TYR), spermidine trihydrochloride (SPD), and spermine tetrahydrochloride (SPM) were individually prepared at 1% and 0.1% concentration in distilled water.

2.3.2. Derivatization of Doenjang extracts and BA solutions

Extraction of BAs from *Doenjang* samples and derivatization of BAs were carried out according to our previously developed method (Shukla et al., 2010). Finally, 1 mL of each derivatized extract from *Doenjang* samples or standard amine solution was filtered through 0.2 mm (pore size) syringe filter and analyzed using HPLC (defined below in 2.3.3). All prepared samples were stored at −25 °C before use and tested by HPLC. Each experiment was performed in triplicate.

2.3.3. High performance liquid chromatography (HPLC) analysis

BAs were quantitatively analyzed using an HPLC system, consisting of two pumps and a UV–vis detector. Separation was achieved using a C18 Water column (Milford, MA, USA) of 4.6 × 250 mm size. The mobile phase was ammonium acetate (0.1 M; solvent A) and acetonitrile (solvent B) at the flow rate of 1 mL/min with gradient elution program for 35 min. The sample volume injected was 10 μL. The samples were monitored at 254 nm. Each HPLC run took about 30 min, after which the column was conditioned again with a mixture of 50% solvent A and 50% solvent B.

2.4. Determination of reduction in B. cereus count in Doenjang samples fortified with plant extracts

To analyze reduction in *B. cereus* count, 10 g of each *Doenjang* sample fortified with plant extracts was homogenized using a blender in 90 mL of 0.85% sterile saline and serially diluted to $10^{-1\sim6}$ with KH_2PO_4 buffer solution (pH 7.2). Enumeration of *B. cereus* was performed by spreading 0.2 mL of each diluted sample onto the surface of mannitol-egg yolk-polymyxin agar (MYP) plates, and the plates were incubated at 30 °C for 24 h (MFDS, 2015). Colonies of pink color with transparent zones were counted, and further confirmatory tests were performed by streaking the observed colonies onto nutrient agar (NA), and grown colonies were further authenticated using an API 50 CHB kit (bioMerieux, Marcy-I'Etoile, France). Cultures showing positive reactions with all assays were confirmed as *B. cereus*. Based on the final results, colonies were further determined quantitatively.

2.5. Enumeration of foodborne pathogens in Doenjang samples fortified with plant extracts

Determination of foodborne pathogenic bacteria, such as *Salmonella* spp., *Staphylococcus aureus*, *Clostridium perfringens, Escherichia coli,* and *coliform* in *Doenjang*, is very important to ensure good food quality and to provide consumers with safe food products. *Salmonella* spp. and *S. aureus* were enumerated by standard methods (MFDS, 2015), while coliforms were enumerated by Sanita-Kun coliform count (SCC, Chisso Corp., Tokyo, Japan).

2.6. Determination of total aflatoxin in Doenjang samples fortified with plant extracts

Extraction of aflatoxin from *Doenjang* samples was performed according to the manufacturer's instructions (Neogen Corp., Lansing, MI, USA) provided with the quantitative aflatoxin test kits (Veratox for total aflatoxin). The results were calculated using the Neogen's Veratox data reduction informatics software.

2.7. Antioxidant potential of Doenjang samples fortified with plant extracts

2.7.1. Total phenolic and flavonoid content and DPPH radical scavenging assay

The total phenolic and flavonoid contents in *Doenjang* samples after 12 months of fermentation were measured spectrophotometrically. The amount of total phenolics and flavonoids was calculated as mg of gallic acid equivalents (GAE/g dry mass) and quercetin equivalents (QE/g dry mass). DPPH assay was carried out as reported previously (Blois, 1958). DPPH radical scavenging ability of *Doenjang* products fortified with plant extracts (EMD and TMD) was calculated at different concentrations (1, 5, and 10 mg/mL) using the following equation, in which H and H0 are the optical densities of the solvent with and without sample, respectively.

$$\text{Radical scavenging activity (\%)} = \{(1–H)/H0\} \times 100$$

2.7.2. Determination of cellular reactive oxygen species (ROS)

Human lung bronchial epithelial cells (BEAS-2B, CRL-9609) (5×10^4 cells/well) were seeded in a 12-well plate until a dense single layer of cells with full confluence growth was observed. Thereafter, cells were treated with TMD GIM1 *Doenjang* samples supplemented with various plant extracts (1%) at different test concentrations (1, 5, and 10 mg/mL). After 24 h of incubation, oxidative stress was induced in the cells by treatment with 0.25 mM H_2O_2 for 2 h. Finally, cells were washed twice with PBS, followed by DCFDA (20 μM) staining. After 30 min incubation, stained cells were washed with PBS and immediately visualized using a fluorescent microscope (Nikon TS 100, Japan). The fluorescence intensity was quantified by the image J software.

2.8. Physico-chemical properties of Doenjang samples fortified with plant extracts

2.8.1. Measurement of pH

For pH measurement, each *Doenjang* sample was diluted 10-fold with distilled water and homogenized followed by filtration with Whatman paper No. 2 (Advantec, Tokyo, Japan); pH was measured using a pH meter (Orion 35 star pH Benchtop, Thermo Electron Corporation, Beverly, MA, USA).

2.8.2. Color analysis

The color values (L*, a* and b*) of *Doenjang* samples were measured with a Chromameter (CR-300; Minolta; Osaka, Japan). *Doenjang* samples were placed on the surface of a white standard plate and the Hunter L, a, and b color values were measured, where L = 96.43, a = +0.03 and b = +1.79. The L* value indicates the lightness, 0–100 representing dark to light (Byun et al., 2013). The a* value gives the degree of the red-green color with a higher positive a*, indicating more red. The b* value indicates the degree of yellow-blue color with higher positive b* value, indicating more yellow.

2.9. Quantification and statistical analysis

Reproducibility was determined by repeated measurements (n = 6) of each *Doenjang* sample. Linearity of known concentrations of standard BAs was tested by regression analysis from three independent calibration curves that contained all the analytes (mixture of standards of biogenic amines). The data were presented as mean and standard deviation of the analyzed samples.

3. Results and discussion

3.1. Fermentation of Doenjang samples fortified with plant extracts

As mentioned earlier, we prepared *Doenjang* samples in two ways in order to confirm the appropriate stage for the addition of the plant extracts during *Doenjang* fermentation: (i) *Doenjang* with *Meju* containing plant extracts (EMD) and (ii) *Doenjang* with traditional *Meju* (TMD), where plant extracts were added during the fermentation process.

In case of EMD samples, steamed soybeans were mixed with plant extracts at 1% and 10% concentration and prepared in square cake shaped *Meju* samples followed by natural drying in open environment with rice straw hanging for 30 days. On the other hand, for TMD samples, traditional *Meju* samples were obtained from traditional market in Korea. After this, the real process of *Doenjang* fermentation was preceded with EMD *Meju* samples by adding 15% of salt water. In case of TMD *Doenjang*, 15% salt water was mixed in each *Meju* cake followed by the addition of the plant extracts at 1% and 10% concentration. *Doenjang* samples were allowed to ferment in natural conditions and samples were divided for fermenting in different time schedules (3, 6, 9 and 12 months), similar to the traditional *Doenjang* preparation procedure (Fig. 2). Subsequently, *Doenjang* samples were collected at the end of each fermentation process, conducted for a specific time period (0, 3, 6, 9 and 12 months) and stored at −4 °C for further studies. All *Doenjang* samples produced were assigned sample codes as mentioned in Table 1.

Fig. 2. Scale-up fermentation for plant extracts added *Meju* and *Doenjang* samples under natural environment, with standardized protocols.

Table 1
List of various *Doenjang* samples prepared with plant extracts (individual & combined).

Meju sample	Plant extracts ratio and composition used for Doenjang	Abbreviation	
		EMD	TMD
1	Doenjang with Garlic extract at 1% concentration	GAD1	GAD1
2	Doenjang with Garlic extract at 10% concentration	GAD10	GAD10
3	Doenjang with lotus extract at 1% concentration	LOD1	LOD1
4	Doenjang with lotus extract at 10% concentration	LOD10	LOD10
5	Doenjang with Ginkgo extract at 1% concentration	GID1	GID1
6	Doenjang with Ginkgo extract at 10% concentration	GID10	GID10
7	Doenjang with mixture extract (1:1:1) at 1% concentration	MID1	MID1
8	Doenjang with mixture extract (1:1:1) at 10% concentration	MID10	MID10
9	Doenjang without extracts (original traditional Meju)	CON	CON

3.2. Determination of BAs in Doenjang samples fortified with plant extracts

The nine most prevalent biogenic amines (TRP, PUT, HIS, TYR, SPM, PHE, AGM, CAD, and SPD) determined in this study were analyzed in TMD and EMD *Doenjang* samples fortified with lotus (LOD), ginkgo (GID), and garlic (GAD) plant extracts as well as controls. All *Doenjang* samples were fortified with 1% and 10% of lotus (LOD1 and LOD10), ginkgo (GID1 and GID10), and garlic (GAD1 and GAD10) plant extracts respectively as well as with a combination of two concentrations (MID1 and MID10). BAs were well resolved and were identified on the basis of their retention times by comparison with their respective standard solutions. The chromatograms of separated BAs are shown in Fig. 3A and B. Among the tested EMD *Doenjang* samples, histamine represented the highest value (0.30 ± 0.14–22.58 ± 25.72 mg/100 g) after 9 months of fermentation, which was found to be reduced after 12 months of fermentation in the range of 0.00 ± 0.00~13.12 ± 0.16 mg/100 g (Table S1). However, in control *Doenjang* sample it was found to be 26.23 ± 1.16. In case of EMD, GID10 showed no histamine after 9 and 12 months of fermentation. Histamine poisoning is a worldwide problem (Russel and Maretic, 1986) that occurs after the consumption of food containing BAs, particularly histamine at concentration higher than 500 ppm (Gonzaga et al., 2009). AGM, TRP, and CAD were not detected in most of the *Doenjang* samples during 0, 3, and 6 months of fermentation, while they were present in negligible amounts (0.00 ± 0.00–3.69 ± 0.35, 0.00 ± 0.00–4.12 ± 4.98 and 0.07 ± 0.09–41.51 ± 47.73, respectively) in 9 months fermented EMD *Doenjang* samples. Even after 12 months of fermentation, the levels of the detected BAs were found to be reduced to negligible levels, confirming that there might be some effect of the added plant extracts on the reduction of BAs in *Doenjang* products during the fermentation process (Fig. 3C).

On the other hand, TMD *Doenjang* samples showed lower levels of

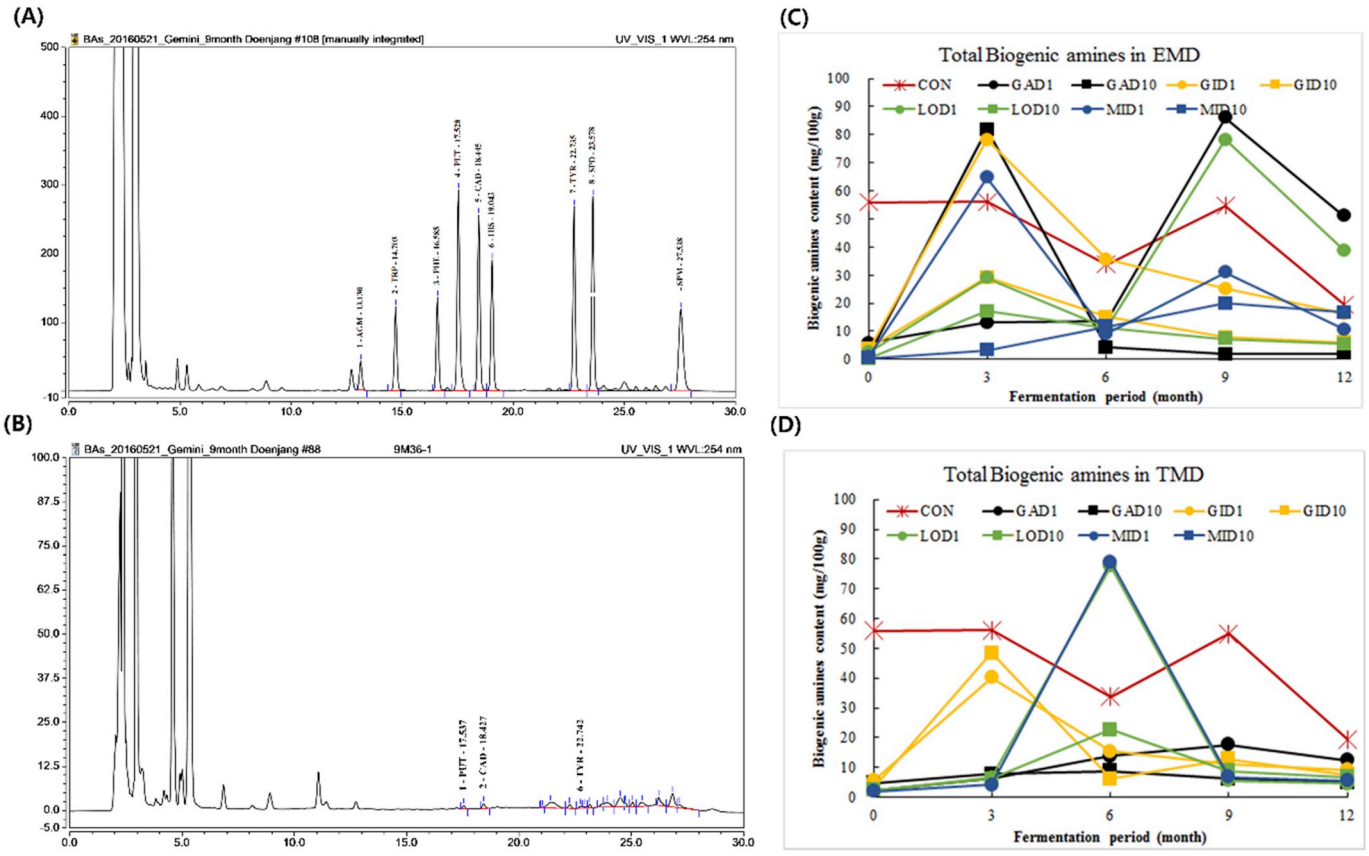


Fig. 3. Biogenic amine measurement in *Doenjang* samples fermented for different time periods (A) Chromatograms for BAs during 6 months of fermentation; (B) Chromatograms for BAs during 6 months of fermentation; (C) Total BAs in EMD *Doenjang* samples, fermented for different time periods; (D) Total BAs in TMD *Doenjang* samples fermented for different time periods.

total BAs after 12 months of fermentation than EMD *Doenjang* samples (4.56 ± 0.04–12.37 ± 2.10) in all tested samples fortified with plant extracts (Table S2). Control *Doenjang* sample after 12 months showed BA level of 39.52 ± 5.78. *Doenjang* samples fortified with plant extracts, in both ways (TMD and EMD) showed lower levels of BAs as compared to the *Doenjang* control sample (which was not fortified with plant extracts), confirming the potential effect of plant extracts in reducing BAs levels in the *Doenjang* samples. Histamine level in *Doenjang* samples fermented for 9 months was lower than EMD samples (0.00 ± 0.00–4.60 ± 4.88 mg/100 g) and similarly, the content of histamine was further reduced after 12 months of fermentation and was in the range of 0.00–4.10 ± 0.02 mg/100 g. These results confirmed that TMD *Doenjang* samples showed higher reduction of BAs than EMD *Doenjang* samples, although in both cases, BAs were controlled as per the limit of standard communities (100 mg/100 g). Moreover, the levels of some of the amines detected in a few *Doenjang* samples after fermentation for 3 and 6 months were found to be reduced after 9 months, and further reduction was observed after 12 months of fermentation (Fig. 3D). Interestingly, BAs content reduced gradually which might be due to the action of the microbial decarboxylase enzymes produced in synergism with the added plant extracts. Enhanced levels of BAs in *Doenjang* samples during 9 months of fermentation might be due to some decarboxylase enzyme activities and some unwanted harmful natural microbial contaminants or cross contamination of initially colonized bacterial species after 6 months of fermentation. However, levels of BAs in *Doenjang* samples were reduced in the 12th month. We assume that after 9 months, the phytoconstituents present in the plant extracts showed inhibitory effects against the contaminants, thus normalizing the levels of BAs in *Doenjang* samples. In case of EMD samples, the amount of BAs during 9 months of fermentation was higher than in TMD *Doenjang* samples where plant extracts were added separately; therefore suggesting the need of using TMD samples with plant extracts. Finally, TMD samples were processed for the development of *Doenjang* products as an innovative approach. Further studies are still in continuation with similar and/or other soybean-based fermented products.

3.3. Reduction of *B. cereus* count/foodborne pathogens in Doenjang samples fortified with plant extracts

In the present study, presumptive test confirmed the reduction of *B. cereus* colonies on MYP agar plates in all prepared *Doenjang* samples (Table 2). All the tested EMD *Doenjang* samples showed either no growth or reduced count of *B. cereus* after 3, 6, 9, and 12 months of fermentation (0.00–2.0 × 10^2 CFU/g) than the control *Doenjang* sample (3.4 × 10^2–7.8 × 10^2 CFU/g) (Fig. 4). All the tested TMD *Doenjang* samples showed high number of colonies of *B. cereus* (1.3 × 10^1–7.9 × 10^2 CFU/g) than the EMD *Doenjang* samples. These results confirmed that *Meju* containing plant extracts used for the production of EMD *Doenjang* was more effective in reducing *B. cereus* counts than traditional *Meju*, which was used for production of TMD *Doenjang*, where plant extracts were added to the *Meju* during the course of fermentation. Nonetheless, *B. cereus* counts of TMD *Doenjang* samples were also within the regulatory limits. Confirmatory tests for *B. cereus* were performed by the molecular identification of the suspected colonies, which showed 100% similarity with other *B. cereus* strains. Further, other hazardous foodborne pathogens (*Salmonella* spp., *S. aureus*, *C. perfringens*, *E. coli* and *coliform*) were not detected in any of the *Doenjang* samples fortified with plant extracts even after 12 months of fermentation.

3.4. Reduction rate of aflatoxin in Doenjang samples fortified with plant extracts

In this study, all the tested *Doenjang* samples (EMD and TMD) showed aflatoxin levels (Table 3) under the hazardous limit, as defined by different authorities. The total aflatoxin level in the majority of the samples was in the range of 1.15 ± 0.54–4.00 ± 1.72 μg/kg after 12 months of fermentation, and was found below the quantitative limit. There was no significant difference in aflatoxin levels based on the duration of the fermentation process (0, 3, 6, 9, and 12 months). Similar aflatoxin level was detected in the control *Doenjang* sample (2.32 ± 1.17 μg/kg). According to the Codex Alimentarius Commission, Joint FAO/WHO food standards program adopted a consumption limit of 15 ppb (15 mg/kg) for total aflatoxins. Thus, all prepared *Doenjang* samples were considered safe for human consumption. It might be possible that the level of aflatoxin was reduced due to individual plant extracts and/or due to their combined effect during *Meju*/*Doenjang* fermentation.

Table 2
Analysis of *Doenjang* samples (fermented for 0, 3, 6, 9, and 12 months) for reduced *B. cereus* counts.

Doenjang samples	*B. cereus* (CFU/g) Presumptive test (MYP[1])				
	0 month	3 month	6 month	9 month	12 month
CON	1.7×10^3	1.8×10^4	1.5×10^3	1.7×10^3	3.4×10^2
GAD1	TMD 1.0×10^2	5.0×10^1	3.1×10^2	2.7×10^2	1.0×10^2
	EMD 1.0×10^1	1.0×10^1	1.0×10^1	1.0×10^1	nd
GAD10	TMD 2.0×10^1	2.0×10^1	1.2×10^2	1.0×10^2	2.1×10^2
	EMD nd	1.0×101	nd	nd	nd
LOD1	TMD 6.0×10^1	5.0×10^1	3.5×10^2	2.4×10^2	1.3×10^1
	EMD 4.0×10^1	4.0×10^1	1.0×10^1	1.0×10^1	nd
LOD10	TMD nd	2.0×10^2	4.6×10^2	3.5×10^2	8.0×10^0
	EMD nd	nd	1.0×10^1	1.0×10^1	nd
GID1	TMD 3.0×10^2	1.9×10^2	5.4×10^3	4.7×10^3	6.4×10^2
	EMD 1.0×10^1	1.0×10^1	nd	nd	1.3×10^1
GID10	TMD nd	2.0×10^2	6.2×10^3	4.1×10^3	1.3×10^1
	EMD nd	nd	nd	nd	1.1×10^2
MID1	TMD nd	nd	1.5×10^4	1.0×10^4	7.9×10^2
	EMD nd	nd	1.0×10^1	nd	7.8×10^2
MID10	TMD 1.0×10^1	2.2×10^1	3.1×10^4	2.1×10^4	5.1×102
	EMD 2.0×10^2	1.0×10^1	1.0×10^1	1.0×10^1	6.5×01

TMD: *Doenjang* produced using traditional *Meju* with addition of plant extracts during fermentation; EMD: *Doenjang* prepared with extract *Meju*; nd: Not detected.

3.5. Antioxidant potential of Doenjang samples fortified with plant extracts

3.5.1. Total phenolic, flavonoid content and DPPH radical scavenging assay

Before selecting the best prepared *Doenjang* sample for animal studies, we examined the antioxidant potential of the samples, which is considered a basic requirement for acceptable functionality of fermented products. We analyzed the total phenolic and flavonoid contents of the *Doenjang* samples, fortified with plant extracts (TMD and EMD) and compared the values with each other, after 12 months of fermentation (Table 4). The total phenolic contents of EMD and TMD samples after 12 months of fermentation were found in the range of 295.09 ± 3.93–359.74 ± 11.51 mg GAE/g and 307.00 ± 3.22–429.17 ± 12.42 mg GAE/g, respectively. The phenolic content of the control samples was 370 ± 11.2 mg GAE/g. In addition, the total flavonoid contents in EMD and TMD samples after 12 months of fermentation were in the range of 71.12 ± 2.37–100.01 ± 4.52 mg QE/g and 78.85 ± 4.65–119.56 ± 4.14 mg QE/g, respectively, while in the control sample it was 65.43 ± 2.85 mg QE/g.

Furthermore, after 12 months of fermentation, all the *Doenjang* samples fortified with plant extracts were analyzed for DPPH radical scavenging activity. The samples were extracted with distilled water, followed by freeze-drying and thereafter reconstituted at different concentrations (1, 5, and 10 mg/mL). DPPH radical scavenging activities of all tested *Doenjang* samples (TMD, EMD) fermented for 12 months with lotus, ginkgo, and garlic extracts, individually and/or in combination (in a ratio of 1:1:1) as well as control *Doenjang* sample (traditional *Doenjang* sample without plant extracts) are presented in Fig. 5. The results confirmed that all tested *Doenjang* samples (EMD and

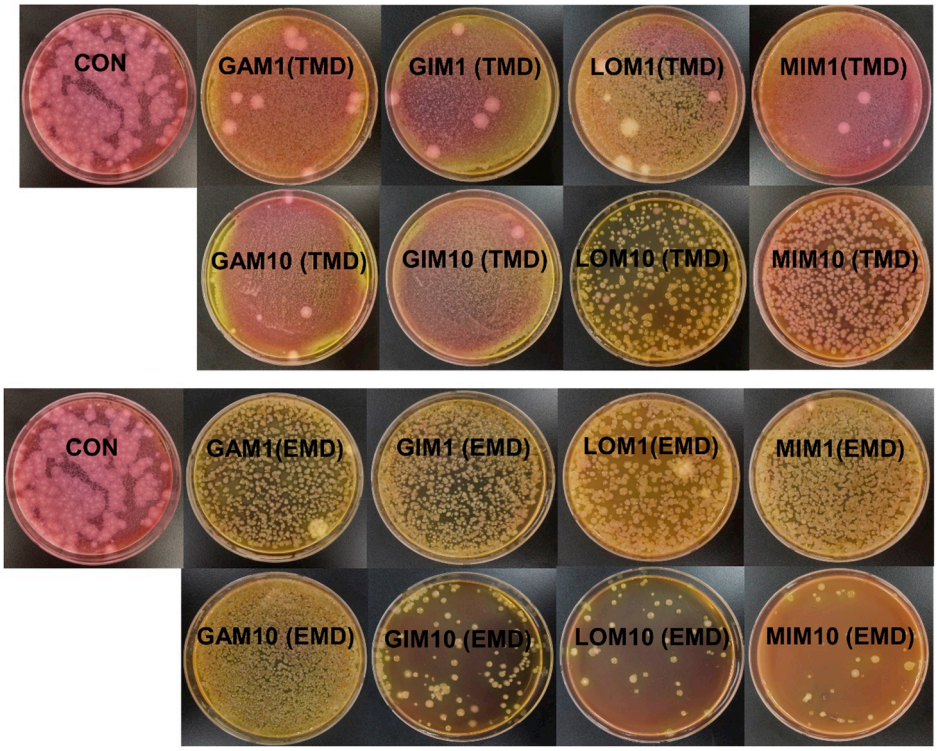


Fig. 4. Presence and absence of *B. cereus* colonies in TMD and EMD *Doenjang* samples.

TMD) fortified with plant extracts (individually and/or in combination) showed higher DPPH radical scavenging activity than control samples. All TMD (lotus, ginkgo, and garlic extracts added) *Doenjang* samples (individually and/or in combinations at 1:1:1 ratio) showed 80.84, 89.38, 84.34, and 91.67% inhibitory activities at a higher concentration (10%) of plant extract GAD10, GID10, LOD10, and MID10, respectively (Fig. 5). Traditional *Doenjang* sample as a control showed 72.85% inhibitory effect at the same concentration. A gradual and significant ($p < 0.05$) elevation of antioxidant activity was observed in all the tested *Doenjang* samples with increasing concentration of plant extracts.

On comparing the difference between TMD and EMD samples, it was noticed that in both cases, LOD, GID, and MID samples showed good antioxidant activities as compared to garlic (GAD) as well as the control (CON) (Fig. 5A and B). Interestingly, the difference observed between 10% TMD and EMD samples was not very significant. Therefore, TMD sample was considered for scale-up production of *Doenjang* samples fortified with plant extracts. The production of TMD samples was more cost effective because it can eliminate the specific process for producing plant extracts added *Meju*. Rather, the desired plant extracts can be added to the normal traditional *Meju* (available in traditional markets) during the course of fermentation, enabling industrial feasibility and fast production.

3.5.2. Cellular antioxidant potential

Since the production process of TMD sample was found feasible for further commercialization, therefore, based on the laboratory sensory panel evaluation, TMD-based GID1 *Doenjang* sample was processed further for confirming the cellular antioxidant potential due to its

Table 3
Determination of aflatoxin in *Doenjang* samples (fermented for 0, 3, 6, 9, and 12 months) prepared with individual plant extracts and their combinations.

Doenjang samples		Total aflatoxin (μg/kg)				
		0 month	3 month	6 month	9 month	12 month
CON		2.48 ± 0.01	2.50 ± 0.15	2.44 ± 0.00	2.51 ± 0.21	2.32 ± 1.17
GAD1	TMD	1.37 ± 0.05	2.05 ± 0.12	1.70 ± 0.01	1.59 ± 0.24	1.25 ± 0.94
	EMD	1.79 ± 0.02	1.54 ± 0.13	1.85 ± 0.05	1.35 ± 0.52	1.32 ± 0.53
GAD10	TMD	1.74 ± 0.13	2.40 ± 1.05	1.50 ± 0.00	1.21 ± 0.00	1.15 ± 0.40
	EMD	1.52 ± 0.01	1.89 ± 0.15	1.75 ± 0.12	1.65 ± 0.14	2.20 ± 1.54
LOD1	TMD	0.53 ± 0.25	0.32 ± 0.02	0.21 ± 0.02	1.62 ± 0.00	2.20 ± 1.10
	EMD	1.01 ± 0.00	1.68 ± 0.52	0.30 ± 0.02	0.91 ± 0.00	2.02 ± 0.43
LOD10	TMD	1.40 ± 0.04	1.63 ± 0.25	1.05 ± 0.00	1.54 ± 0.16	1.50 ± 0.68
	EMD	1.32 ± 0.13	1.37 ± 0.25	1.40 ± 0.00	1.52 ± 0.21	2.02 ± 0.88
GID1	TMD	1.93 ± 0.52	1.62 ± 0.25	1.17 ± 0.05	2.03 ± 0.00	1.00 ± 1.72
	EMD	1.27 ± 0.22	1.67 ± 1.05	1.30 ± 0.05	2.06 ± 0.21	2.05 ± 0.66
GID10	TMD	2.01 ± 0.00	2.17 ± 1.52	1.35 ± 0.00	1.28 ± 0.15	1.02 ± 0.17
	EMD	1.27 ± 0.28	1.98 ± 0.15	1.10 ± 0.02	1.68 ± 0.12	2.15 ± 0.54
MID1	TMD	2.01 ± 0.00	1.93 ± 0.01	1.47 ± 0.02	1.83 ± 0.31	1.30 ± 0.40
	EMD	1.27 ± 0.35	2.75 ± 0.25	1.75 ± 0.02	1.68 ± 0.21	1.61 ± 0.65
MID10	TMD	1.52 ± 0.01	2.01 ± 0.01	1.29 ± 0.00	1.19 ± 0.91	1.25 ± 0.38
	EMD	2.51 ± 0.15	2.79 ± 1.05	1.95 ± 0.00	2.01 ± 0.22	1.75 ± 0.38

TMD: *Doenjang* produced using traditional *Meju* and addition of plant extracts during fermentation; EMD: *Doenjang* prepared with extract *Meju*.

Table 4
Total phenolic and flavonoid contents in TMD and EMD *Doenjang* samples.

Total phenolic contents (mg/GAE/g)			Total flavonoid contents (mg/QE/g)	
Samples	EMD	TMD	EMD	TMD
CON	370.69 ± 11.2^{a}	370.00 ± 11.2^{e}	65.43 ± 2.85^{h}	65.43 ± 2.85^{i}
GAD1	311.186 ± 6.47^{f}	331.23 ± 7.11^{h}	75.67 ± 3.00^{f}	79.87 ± 1.38^{e}
GAD10	307.99 ± 5.84^{g}	307.00 ± 3.22^{i}	82.25 ± 2.54e	78.85 ± 4.65^{f}
GID1	295.09 ± 3.93^{h}	377.28 ± 6.88^{d}	71.12 ± 2.37^{g}	68.77 ± 2.97^{h}
GID10	350.81 ± 7.75^{c}	380.31 ± 4.36^{c}	83.98 ± 4.18^{d}	75.74 ± 2.64^{g}
LOD1	322.36 ± 5.60^{e}	429.17 ± 12.42^{a}	90.39 ± 2.36^{b}	119.56 ± 4.14^{a}
LOD10	359.74 ± 11.51^{b}	353.16 ± 4.36^{f}	100.01 ± 4.52^{a}	93.28 ± 4.56^{c}
MID1	342.58 ± 5.11^{d}	422.43 ± 4.64^{b}	90.02 ± 2.59^{b}	115.88 ± 2.32^{b}
MID10	308.35 ± 4.90^{g}	334.76 ± 5.45^{g}	89.8 ± 2.59^{c}	90.03 ± 2.77^{d}

TMD: *Doenjang* produced using traditional *Meju* and addition of plant extracts during fermentation; EMD: *Doenjang* prepared with extract *Meju*. Superscripts sharing a common letter in the same column are not significantly different at $P < 0.05$ by Duncan's multiple range test.

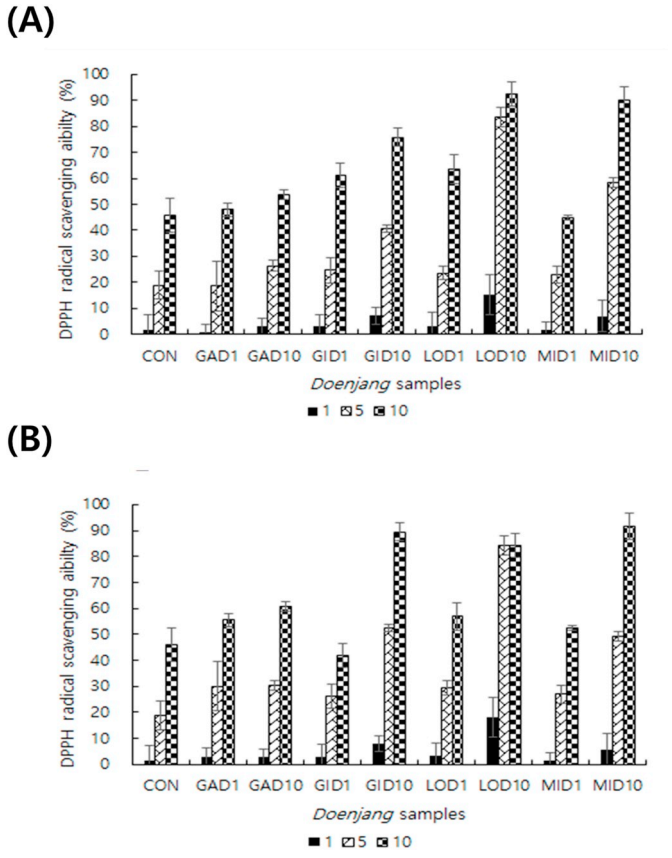


Fig. 5. Effect of EMD and TMD *Doenjang* samples fortified with plant extracts on DPPH radical scavenging ability.

acceptable color and taste values similar to the traditional *Doenjang* samples. Results of cellular antioxidant activity evaluated by DCFDA staining revealed a dose-dependent effect of TMD GID1 *Doenjang* on the inhibition of ROS production in human lung epithelial cells, challenged by H_2O_2 (ROS inducer). A dose-dependent decrease in the fluorescent intensity was observed in the cells treated with 1, 5, and 10 mg/mL concentration of TMD GID1 sample as 90.01 ± 10.21, 65.50 ± 5.51, 45.67 ± 5.41%, respectively, as compared to 100%, in only H_2O_2 stimulated cells (Fig. 6A and B). Previous reports suggest the abundance of phenolics and flavonoids in various soy fermented foods (Lin et al., 2006), and we speculate that the cellular antioxidant activity of TMD-GID1 could be due to the presence of these diverse phytochemicals in it. To the best of our knowledge, till date, no study has demonstrated the cellular antioxidant nature of *Doenjang*. However, a few studies have shown the antioxidant potential of *Doenjang in vitro* (Kim et al., 2018).

3.6. Measurement of pH and color values of Doenjang samples fortified with plant extracts after 12 months fermentation

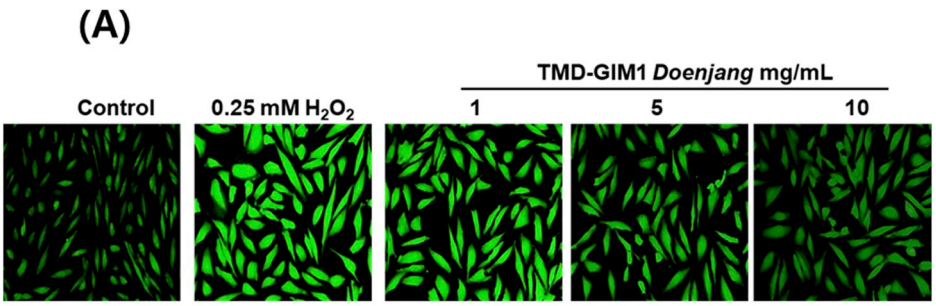
No significant difference in pH values was observed among the different *Doenjang* samples prepared by fermentation for different time periods. The pH of *Doenjang* samples (with prior addition of plant extracts) fermented for 12 months ranged from 5.39 to 6.05. *Doenjang* samples (without plant extracts) fermented traditionally showed a pH of 6.04. Shukla et al. (2014) also observed a similar range of pH in *Doenjang* samples fermented with the starter culture. The pH values were found to decrease with increase in fermentation time. Fermented foods are usually considered safe below pH 4 to 6 as most pathogens are unable to survive at this pH range (Padonou et al., 2010).

Doenjang samples (TMD and EMD) showed significant differences in color quality, which might be due to the use of different plant extracts at different concentrations (1% and 10%). Inner proportions of TMD and EMD *Doenjang* samples showed similar color values for yellow color for each fermentation period, which might be due to the Milliard reaction occurring during fermentation, since soybeans contain several amino acids and sugars. In addition, other components of the added plant extracts may also be responsible for the browning reactions (Fig. 7 and Table S3).

Several bio-control approaches, such as use of bacteria, yeast, and fungi as starter culture (Alberts et al., 2006), have been approved to reduce the biological contaminants. However, in the present study, we used single and combined plant extracts for reducing BA content, aflatoxins, and foodborne pathogenic bacteria, including *B. cereus,* in *Doenjang* samples. Overall, in the present study, the results obtained from the *Doenjang* samples prepared with plant extracts, showed reduction in BAs content, aflatoxins, and foodborne pathogenic bacteria, including *B. cereus,* compared to the original traditional *Doenjang* samples (control) prepared without addition of plant extracts. Moreover, apart from the reduction in *B. cereus* counts, *Doenjang* samples prepared with plant extracts showed absence of foodborne pathogenic bacteria, including *Salmonella* spp., *S. aureus*, *C. perfringens*, *E. coli* and *coliform* until 9 months of fermentation. The research team of our laboratory examined the taste and flavor of all *Doenjang* samples produced, and categorized them: GID1 and GAD1 (very good); GID10 and MID1 (not bad), GAD1 and GAD10 (good); and LOD1, LOD10 and MID10 (not good).

4. Conclusion

In the present study, we determined the specific stages, appropriate for addition of selected plant extract-based starter or plant extracts during the startup fermentation procedure. Overall, it was found that all prepared *Doenjang* samples (TMD and EMD) showed desired reduction in *B. cereus* counts, BAs, aflatoxins, and other foodborne pathogens. Reduction in the levels of these toxins (BAs) was found to be



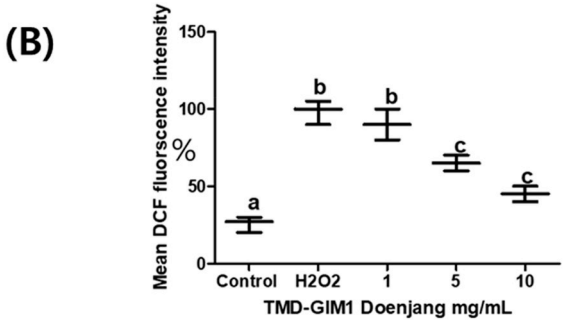


Fig. 6. Effect of *Doenjang* sample TMD GIM1 on reactive oxygen species in human lung epithelial cells, challenged by H_2O_2. DCFDA staining (I) without H_2O_2 and GID1 (II) Only 0.25 mM H_2O_2 (III) 1 mg/mL GID1 + 0.25 mM H_2O_2 (IV) 5 mg/mL GID1 + 0.25 mM H_2O_2 (V) 10 mg/mL GID1 + 0.25 mM H_2O_2. All images were taken by an EPI fluorescence microscope at 40 × magnification [Scale bar = 0.1 mm]. Fluorescence intensity was determined using Image J software. Each value represents mean ± standard deviation (SD) of three independent experiments.

most pronounced at 12 months of fermentation, which confirms that these selected plant extracts played a significant role in the reduction of these hazardous toxins. Based on the advised sensory characteristics, consumer acceptability, and functionality point of view, *Doenjang* samples fortified with *Ginkgo biloba* extract were selected for further product development. By comparing EMD and TMD *Doenjang* samples, the TMD *Doenjang* sample fortified with ginkgo, was further processed due to its easy production feasibility, which eliminated the use of any specially prepared *Meju* samples. By developing this new *Doenjang* product with reduced *B. cereus* counts, aflatoxin, and BA contents, we may be able to enhance the bio-functionality of the fermented products, driven by the synergistic effects of these plant extracts, when used in

Fig. 7. Inner colors of TMD and EMD *Doenjang* samples fermented with or without plant extracts. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

combination. Moreover, in future, the scale up *Doenjang* production process could be optimized for its commercialization as an innovative *Ginkgo biloba*-based *Doenjang* product, which is a safer alternative for commercialization.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110729.

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