

## Antibacterial, Free Radical Scavenging, and Proliferative Effects of Korean Fermented Soybean Paste (*Doenjang*) Extracts

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Received September 2, 2005; Accepted September 16, 2005

**Antibacterial, free radical scavenging, proliferative, and cytotoxicity effects of *Doenjang* extracts were examined. All samples except water extract showed strong antibacterial activity against oral bacteria, *Streptococcus pyogenes*, *S. mutants*, *S. sanguinis*, *S. sobrinus*, *S. criceti*, *S. aninosus*, *S. gordonii*, and *Porphyromonas gingivalis* (MIC and MBC values: 0.08-1.25 and 0.16-2.50 mg/ml, respectively). DPPH method showed ethanol and ethyl acetate extracts are effective inhibitors of oral bacteria. Based on MTT assay, 24 h exposure to 0.31 mg/ml of all extracts, excepted water extract, resulted in strong cytotoxicity on KB cells. All extracts strongly inhibited human gingival fibroblast viability.**

**Key words:** fermented food, soybean paste, antimicrobial activity, radical-scavenging, cytotoxicity, gingival fibroblast

Great interest has been devoted in recent years to the fermented foods as one of the longevity diets in the world. In particular, special focus has been placed on fermented soybean foods made traditionally in Asian countries for their functional qualities. *Doenjang*, a fermented soybean paste, is one of the important traditional fermented foods in Korea. *Doenjang*, manufactured naturally using a rectangular shape *meju* made with fermented steamed soybean, is a unique soybean food fermented by diverse microorganisms including bacilli and fungi.<sup>1,3)</sup> It is well known to have anticancer, antimutagenic, and antihypertensive activities,<sup>4-16)</sup> and is known to be safe, because aflatoxins produced by *Aspergillus flavus* are eradicated by NH<sub>3</sub>, sunlight, melanoidin formation, and charcoal etc. during the fermentation process.<sup>15-18)</sup>

Many researches reported on the significant anti-carcinogenesis of the functional components of soybean foods,<sup>8-10)</sup> among which the peptide fractions obtained from *Doenjang* and *Chungkook-Jang* were reported to suppress ADP-induced platelet aggregation.<sup>11-13)</sup> Also the active compounds identified as isoflavones, and the water, hexane and ethanol extracts obtained from *Doenjang* are correlated with phytoestrogen, antitumor, antioxidative and antihypertensive functions.<sup>4-23)</sup>

However, although these functions of *Doenjang* have been extensively studied, little is known about the effects on oral microorganisms, which are associated with human periodontal disease such as oral infection, juvenile periodontitis, and dental plaque.<sup>24,25)</sup> Therefore, in this study, antimicrobial, free radical scavenging, proliferation, and cytotoxic activity of *Doenjang* extracts obtained by various polar and non-polar solvents were investigated.

### Materials and Methods

**Preparation of *Doenjang* extracts.** *Doenjang* extracts were prepared by decocting the commercial *Doenjang* samples with 10 times (v/w) water, ethanol, ethyl acetate, *n*-butanol, chloroform, and hexane for 1.5 h. After the extracts was filtered ( $\phi$  0.45  $\mu$ m, Advantec Co., Japan), the residue was boiled for additional 1 h. The filtrates were then combined, lyophilized by freeze dryer, and kept at 4°C until use. The average yield of all extracts was about 34.5% of dried weight.

**Microbial strains.** Antimicrobial activities of water, ethanol, ethyl acetate, *n*-butanol, chloroform, and hexane extracts of *Doenjang* against oral bacteria and reference strains were determined by the broth dilution method. The oral bacterial strains used were: *Streptococcus mutans* ATCC 25175, *S. sanguinis* ATCC 10556, *S. sobrinus* ATCC 27607, *S. ratti* KCTC (Korean collection for type cultures) 3294, *S. criceti* KCTC 3292, *S. anginosus* ATCC 31412, *S. gordonii* ATCC 10558, *Actinobacillus actinomycetemcomitans* ATCC 43717, *Fusobacterium nucleatum* ATCC 10953, *Prevotella intermedia* ATCC 25611, and *Porphyromonas gingivalis* ATCC 33277. The reference strains used were: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *S. epidermidis* ATCC 12228, and *S. pyogenes* ATCC 21059. Brain-Heart Infusion broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI, USA) was used for all bacterial strains except *P. intermedia* and *P. gingivalis*, for which Brain-Heart Infusion broth containing hemin (5  $\mu$ g/ml) and menadione (1  $\mu$ g/ml) was used.

**Minimum inhibitory concentrations/Minimum bactericidal concentrations assay.** Minimum inhibitory concentrations (MICs) of water, ethanol, ethyl acetate, *n*-butanol, chloroform,

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and hexane extracts of *Doenjang* were determined using the broth dilution method in triplicates, and antibacterial activities were examined after incubation at 37°C for 18 h (facultative anaerobic bacteria), 24 h (microaerophilic bacteria), and 1-2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MIC plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of the essential oil that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin and gentamicin were used as standard antibiotics to compare the sensitivity of *Doenjang* extracts against test bacteria.

**Free radical scavenging activity.** Free radical scavenging potentials were tested in a DPPH (1,1-diphenyl-2-picrylhydrazyl) methanol solution.<sup>26)</sup> The degree of decoloration of the solution indicates the scavenging efficiency of the added substance. For water, ethanol, ethyl acetate, *n*-butanol, chloroform, and hexane extracts of *Doenjang*, 1 ml of samples in methanol

was added to 2 ml DPPH solution (10 mg/L), and the absorbance was measured at 517 nm after 5 min. A reference sample was prepared using 1 ml methanol. The antiradical activity was calculated as a percentage of DPPH decoloration using the following equation.<sup>26)</sup>

$$\text{Scavenging effect (\%)} = \left[ \frac{A_{517\text{nm}} \text{ of control} - A_{517\text{nm}} \text{ of sample}}{A_{517\text{nm}} \text{ of control}} \right] \times 100.$$

**Cell culture.** KB cells, a human oral epithelial cell line derived from a human oral epidermoid carcinoma (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone. KB cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Human gingival fibroblasts (HGF) were obtained from explants collected during oral surgery from a healthy patient after consent and prepared as described previously.<sup>27)</sup>

**Table 1. MICs and MBCs (mg/ml) of *Doenjang* extracts against oral bacteria and reference strains**

| Strains                                       | MICs/MBCs (mg/ml) |           |           |           |           |           |                            |                          |
|---|-------------------|-----------|-----------|-----------|-----------|-----------|----------------------------|--------------------------|
|   | H <sub>2</sub> O  | EtOH      | EA        | BuOH      | CH        | Hex       | Ampicillin                 | Gentamicin               |
| <i>E. coli</i><br>ATCC 25922                  | 40<               | 20/40     | 10/20     | 20/40     | 20/40     | 10/20     | 256/256×10 <sup>-3</sup>   | 8/16×10 <sup>-3</sup>    |
| <i>S. aureus</i><br>ATCC 29213                | 40<               | 20/20     | 20/20     | 40/40     | 20/40     | 20/20     | 16/16×10 <sup>-3</sup>     | 2/4×10 <sup>-3</sup>     |
| <i>S. epidermidis</i><br>ATCC 12228           | 40                | 40/40     | 20/20     | 20/40     | 20/20     | 10/20     | 32/64×10 <sup>-3</sup>     | 1/2×10 <sup>-3</sup>     |
| <i>S. pyogenes</i><br>ATCC 21059              | 40<               | 0.32/0.64 | 0.16/0.64 | 0.32/0.63 | 0.64/1.25 | 0.08/0.16 | 4/8×10 <sup>-3</sup>       | 8/16×10 <sup>-3</sup>    |
| <i>S. mutans</i><br>ATCC 25175                | 40<               | 0.08/0.16 | 1.25/5    | 1.25/2.5  | 1.25/2.5  | 1.25/2.5  | 4/4×10 <sup>-3</sup>       | 8/8×10 <sup>-3</sup>     |
| <i>S. sanguinis</i><br>ATCC 10556             | 40<               | 0.32/0.64 | 0.04/0.08 | 0.08/0.16 | 0.16/0.64 | 0.08/0.32 | 32/32×10 <sup>3</sup>      | 8/16×10 <sup>-3</sup>    |
| <i>S. gordonii</i><br>ATCC 10558              | 40                | 0.08/0.08 | 0.08/0.08 | 0.04/0.8  | 0.08/0.16 | 0.08/0.16 | 1/2×10 <sup>-3</sup>       | 2/4×10 <sup>-3</sup>     |
| <i>S. criceti</i><br><sup>a</sup> KCTC 3292   | 40<               | 0.32/0.64 | 0.16/0.32 | 0.08/0.16 | 0.08/0.16 | 0.08/0.16 | 4/4×10 <sup>-3</sup>       | 8/8×10 <sup>-3</sup>     |
| <i>S. ratti</i><br>KCTC 3294                  | 40<               | 1.25/2.5  | 0.16/0.32 | 0.32/0.64 | 0.08/0.16 | 0.08/0.16 | 4/4×10 <sup>-3</sup>       | 4/8×10 <sup>-3</sup>     |
| <i>S. sobrinus</i><br>ATCC 27607              | 40<               | 0.32/1.25 | 0.32/0.32 | 0.32/0.64 | 0.64/1.25 | 0.32/0.64 | 2/2×10 <sup>-3</sup>       | 4/8×10 <sup>-3</sup>     |
| <i>S. anginosus</i><br>ATCC 31412             | 40<               | 0.64/1.25 | 0.63/0.63 | 0.64/1.25 | 40/40     | 10/20     | 4/4×10 <sup>-3</sup>       | 16/16×10 <sup>-3</sup>   |
| <i>A. actinomycetemcomitans</i><br>ATCC 43717 | 40≤               | 10/10     | 2.5/5     | 5/10      | 1.25/5    | 5/5       | 64/64×10 <sup>-3</sup>     | 2/2×10 <sup>-3</sup>     |
| <i>F. nucleatum</i><br>ATCC 51190             | 40<               | 5/10      | 5/5       | 5/10      | 5/10      | 5/10      | 0.25/0.25×10 <sup>-3</sup> | 16/32×10 <sup>-3</sup>   |
| <i>P. intermedia</i><br>ATCC 49046            | 40<               | 5/10      | 5/10      | 5/5       | 5/5       | 5/5       | 32/32×10 <sup>-3</sup>     | 0.5/1×10 <sup>-3</sup>   |
| <i>P. gingivalis</i><br>ATCC 33277            | 40<               | 0.64/1.25 | 0.32/0.63 | 0.64/1.25 | 0.32/0.64 | 0.32/0.32 | 0.5/1×10 <sup>-3</sup>     | 256/512×10 <sup>-3</sup> |

<sup>a</sup>Korean Collection for Type Cultures

H<sub>2</sub>O: water extract; EtOH: ethanol extract; EA: ethyl acetate extract; BuOH: *n*-butanol extract; CH: chloroform extract; Hex: hexane extract

**Table 2. Scavenging effects (%) of *Doenjang* extracts on 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals**

| mg/ml | Scavenging effects (%) |                |                |                |                |                |
|-------|------------------------|----------------|----------------|----------------|----------------|----------------|
|       | H <sub>2</sub> O       | EtOH           | EA             | BuOH           | CH             | Hex            |
| 0     | 0.89 ± 0.655           | 0.53 ± 0.152   | 0.64 ± 0.527   | 0.71 ± 0.799   | 1.00 ± 0.587   | 0.85 ± 0.447   |
| 0.01  | 6.60 ± 0.756           | 7.80 ± 2.897   | 4.28 ± 1.369   | 7.84 ± 2.646   | 5.45 ± 0.281   | 5.41 ± 0.442   |
| 0.02  | 11.6 ± 3.683           | 10.14 ± 2.673  | 6.29 ± 3.072   | 11.0 ± 3.001   | 7.37 ± 3.327   | 6.22 ± 0.952   |
| 0.04  | 16.3 ± 4.145           | 11.6 ± 2.624   | 10.9 ± 1.643   | 18.0 ± 3.159   | 9.01 ± 1.992   | 6.65 ± 0.484   |
| 0.08  | 21.5 ± 4.774           | 22.8 ± 2.306   | 16.8 ± 4.002   | 23.4 ± 2.087   | 11.5 ± 2.828   | 8.63 ± 0.377   |
| 0.16  | 31.3 ± 3.706*          | 30.5 ± 4.256*  | 26.6 ± 2.741   | 32.3 ± 1.876*  | 16.4 ± 3.173   | 9.39 ± 0.499   |
| 0.31  | 40.3 ± 4.975**         | 40.2 ± 5.172** | 35.0 ± 1.625*  | 38.6 ± 2.616*  | 21.7 ± 2.360   | 12.3 ± 2.321   |
| 0.64  | 44.2 ± 4.733**         | 52.3 ± 3.821** | 44.6 ± 2.002** | 45.1 ± 2.129** | 29.6 ± 3.159   | 14.3 ± 2.017   |
| 1.25  | 53.0 ± 5.630**         | 57.6 ± 3.906** | 52.4 ± 3.246** | 53.1 ± 1.898** | 39.0 ± 2.178** | 15.1 ± 1.451   |
| 2.5   | 64.5 ± 2.831**         | 65.8 ± 2.768** | 56.8 ± 2.506** | 56.4 ± 0.642** | 45.0 ± 2.767** | 38.3 ± 0.927** |
| 5     | 82.9 ± 1.132**         | 84.6 ± 0.935** | 65.0 ± 2.332** | 65.6 ± 1.629** | 53.2 ± 2.874** | 53.2 ± 2.651** |

The results are expressed as means ± S.D. from three separate experiments with triplicate cultures. \* $p < 0.05$  and \*\* $p < 0.01$  compared with control.

H<sub>2</sub>O: water extract; EtOH: ethanol extract; EA: ethyl acetate extract; BuOH: *n*-butanol extract; CH: chloroform extract; Hex: hexane extract

The cells of KB and HGF were cultured with DMEM supplemented with 10% FBS, 100 U/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone, and the cells of KB and HGF between passages 2 and 6 were used.

**Cell viability.** Cell viability and cytotoxicity were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) staining.<sup>28)</sup> Briefly, HGF and KB cells were plated at 10<sup>5</sup> cells/well into 24-well plates and treated with different concentrations of *Doenjang* extracts for 24 h. After phosphate buffered saline (PBS) washing, the medium was replaced with one containing 100 µl MTT (5 mg/ml), and the cells were incubated for additional 4 h. The resultant purple crystals, produced from the reduction of MTT by metabolically active cells, were then solubilized by DMSO (dimethyl sulfoxide) and left standing for a few minutes at room temperature to ensure that all the crystals were dissolved. The plates were read on a microplate reader (Molecular Devices Sunnyvale Ca. USA) at 540 nm.

**Statistical analysis.** Data are presented as the means and S.E. for the indicated number of separate experiments. Statistical analysis of data was performed through one-way analysis of variance (ANOVA) followed by a *t*-test, and *p*-value less than 0.05 were considered significant.

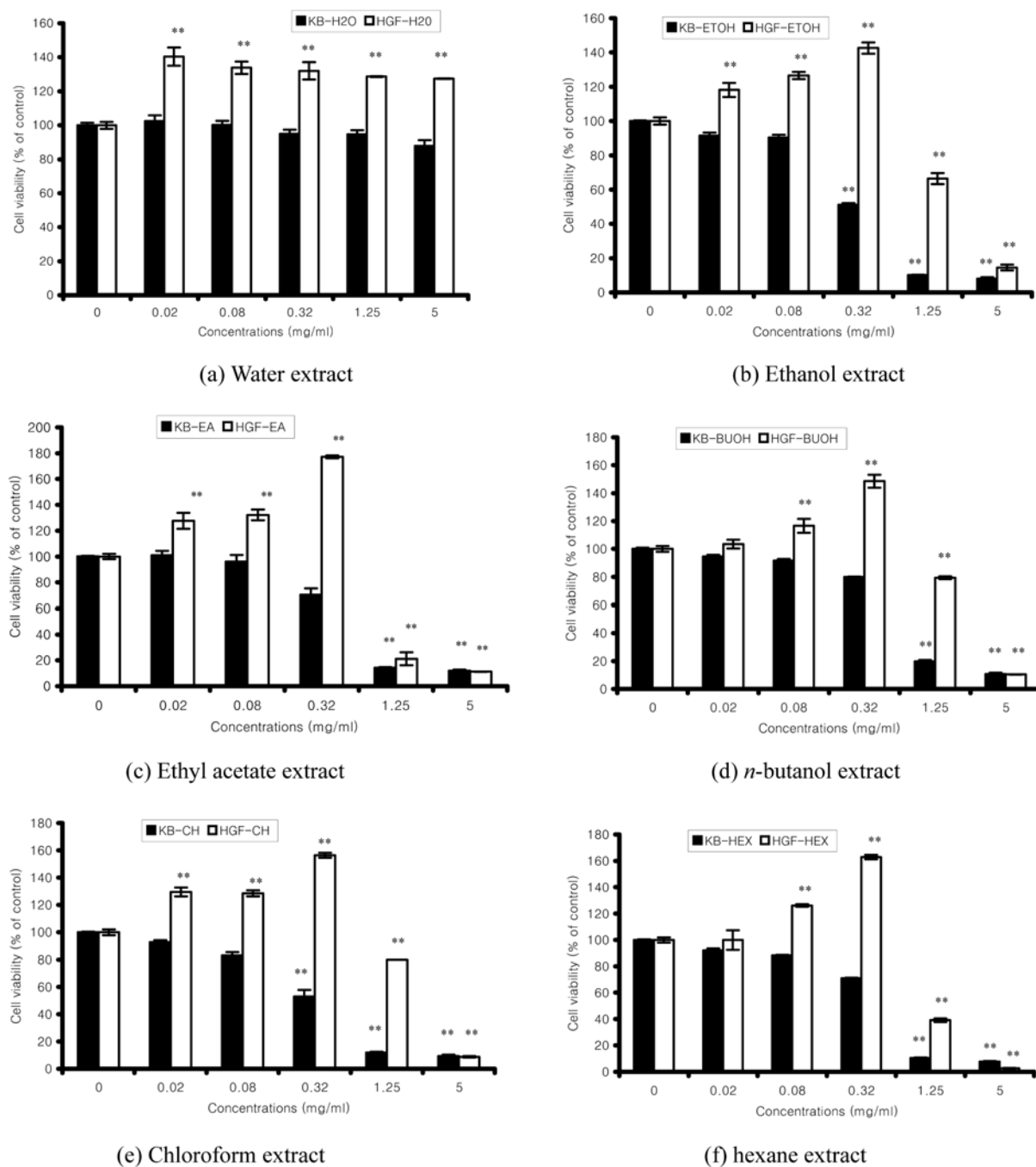
## Results and Discussion

**Antimicrobial activity.** Results of the antibacterial activity (Table 1) showed that the ethanol and ethyl acetate *Doenjang* extracts exhibited moderate activities against all tested bacteria (MIC and MBC values: 0.04-40 and 0.16-40 mg/ml, respectively), and showed strongest activities against the facultative anaerobic bacteria, *S. pyogenes*, *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. criceti*, *S. anginosus*, and *S. gordonii* (MIC and MBC values: 0.08-0.64 and 0.16-1.25 mg/ml, respectively), and obligate anaerobic bacteria, *P. gingivalis*

(MIC and MBC values: 0.64-1.25 and 0.1-0.4 mg/ml, respectively), whereas *E. coli*, *S. aureus*, *S. epidermidis*, *F. nucleatum*, and *P. intermedia* appeared to be the most resistant. The similar observations of possessing antimicrobial activity of *ChungkookJang* against Gram-positive and -negative bacteria, and the *Doenjang*-derived isoflavone against antibiotic-resistant strains including *Staphylococcus aureus* and *S. epidermidis* have been reported.<sup>29,30)</sup>

**Free radical scavenging activity.** The stable free radical DPPH was used to measure the radical-scavenging activities of *Doenjang* extracts. Significant differences were observed in the free radical scavenging activities of all extracts in a dose-dependent manner ( $p < 0.05$ ). The scavenging effect of H<sub>2</sub>O, EtOH, EA, BuOH, CH, and Hex extracts on DPPH at 5 mg/ml was 82.9, 84.6, 65, 65.0, 53.2, and 53.2%, respectively (Table 2), with the ethanol and hexane extracts showing the highest and lowest free radical scavenging activities, respectively. However, the free radical scavenging activities between the fractions of water and ethanol, ethyl acetate and butanol, and chloroform and hexane were not significantly different. Free radical scavenging activities of extracts of soybean and its products on DPPH have been reported as relative inhibition percentages.<sup>2,728)</sup>

**Effect of *Doenjang* extracts on viability of HGF and KB cells as measured by MTT assay.** HGF and KB cells were treated with *Doenjang* extracts at different concentrations for 24 h, and the cell viability was determined by MTT assay. All extracts except the water extract inhibited the growth of KB cells in a dose-dependent manner (Fig. 1). Treatment of MCF-7, human breast cancer cell, with *Doenjang* hexane fraction significantly inhibited pRB (Retinoblastoma protein) phosphorylation and increased Cdk (cyclin dependent kinase) inhibitor p21, which appeared to be responsible for the observed G1-phase of cell cycle arrest.<sup>14)</sup> *Doenjang* extracts and some of its compounds inhibited the growth of human



**Fig. 1.** Effect of water extract (a) and various solvent extracts (b~f) of *Doenjang* on cell proliferation in human gingival fibroblasts (HGF) and KB cells. HGF and KB cells were plated onto 24-well plates and treated with different concentrations of the water extract for 24 h. Cell proliferation was determined by the MTT assay and expressed as percentage of the absorbance value obtained without water extract. The results are expressed as means  $\pm$  S.E. from three separate experiments with triplicate cultures. \*\* $p < 0.01$  compared with control.

cancer cells and induced apoptosis of cancer cells.<sup>10,22)</sup> KB cell viability was inhibited at 0.32 mg/ml in the ethanol and chloroform extracts ( $p < 0.01$ ) (Fig. 1-c, 1-e). HGFs showed strong proliferation at low concentrations (0.02, 0.08, and 0.32 mg/ml) of *Doenjang* extracts for 24 h, whereas induced cytotoxicity at up to 1.25 mg/ml *Doenjang* extracts except the water extract, which did not exhibit cytotoxicity nor mutagenicity at high concentrations.<sup>15)</sup>

In conclusion, the results indicate the ethanol and ethyl acetate extracts of *Doenjang* could be exploited as effective inhibitors of oral bacteria and KB cells, and that all extracts of *Doenjang* are effective proliferators. The amount of *Doenjang* generally used in foods has already been established as safe. Even though more precise efficiency and safety data are required to accurately evaluate the amount of *Doenjang* that could be used for preventive or therapeutic purpose of some

types of cancers. The results suggest that *Doenjang* can be a candidate for antibacterial and anticancer agents.

### Acknowledgments

This research was supported in part by Research Center for Industrial Development of Biofood Materials in Chonbuk National University, Chonju, Korea. The center is designated as a Regional Research Center appointed by the Korea Science and Engineering Foundation (KOSEF), Jeollabuk-Do Provincial Government and Chonbuk National University.

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