Journal of Food Safety

Journal of Food Safety ISSN 1745-4565

THE COMBINED EFFECT OF UV IRRADIATION AND ETHANOL EXTRACT FROM *TORILIS JAPONICA* FRUIT ON INACTIVATION OF *BACILLUS SUBTILIS* SPORES

WON-IL CHO[1]*, CHAN-ICK CHEIGH[2]*, MYUNG-SUB CHUNG[3], KI-HWAN PARK[3], PAHN-SHICK CHANG[4] and MYONG-SOO CHUNG[2,5]

[1]CJ Foods R&D, CJ Corp., Seoul, Korea
[2]Department of Food Science and Engineering, Ewha Womans University, Seoul 120-750, Korea
[3]Department of Food Science and Technology, Chung-Ang University, Anseong, Korea
[4]Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-742, Korea

[5]Corresponding author. TEL: +82-2-3277-4508; FAX: +82-2-3277-4508; EMAIL: mschung@ewha.ac.kr

*These authors contributed equally to this work.

Received for Publication June 8, 2012
Accepted for Publication October 9, 2012

doi: 10.1111/jfs.12010

ABSTRACT

The purpose of this study was to evaluate the synergistic sporicidal effects of the ethanol extract from *Torilis japonica* fruit on inactivation of *Bacillus subtilis* spores induced by UV irradiation at a wavelength of 254 nm. UV_{254} exhibited the strongest inactivation effect, with the level of spores being reduced by approximately 3.6- and 4.7-log cycles, respectively, after 3 and 10 min of exposure at an irradiation distance of 15 cm. And an ethanol extract of *T. japonica* fruit at concentration of 1.0% reduced the spore counts by about 3.0-log cycles for 1 h of treatment. Furthermore, a combined treatment with UV_{254} irradiation and 1.0% ethanol extract of the fruit produced 4.2–5.0-log reductions of *Bacillus* spores at different growth stages for an exposure time of 3 min. The energy required from UV irradiation to inactivate the spores was 50% lower in the presence of 1% ethanol extract of the fruit than with UV irradiation alone.

PRACTICAL APPLICATION

Bacterial endospores, especially those of *Bacillus* and *Clostridium* genera, are the target of sterilization in various foods. The present study suggests that a combined treatment of UV irradiation and natural antimicrobial component, *Torilis japonica* fruit extract, can act synergistically and effectively to kill *Bacillus* spores and can be a potential nonthermal sterilization method for the decontamination of *Bacillus* spores in the food industry.

INTRODUCTION

Attention has recently been focused on the use of nonthermal sterilization methods that employ physical energy such as high pressure, ultrasonic energy and high-voltage electric fields to inactivate the foodborne enteropathogenic bacteria and spoilage fungi responsible for the deterioration of food quality and safety (Marquez *et al.* 1997; Hayakawa *et al.* 1998; Raso *et al.* 1998; Wuytack *et al.* 1998; Shearer *et al.* 2000; Pol *et al.* 2001; Spilimbergo *et al.* 2002). However, endospore-forming bacteria such as *Bacillus subtilis* and *Clostridium botulinum* are resistant to physical treatments. Many novel methods have recently been studied in attempts to overcome the limitations of nonthermal sterilization methods resulting from this resistance. Killing of bacterial spores using pulsed high-voltage electric fields has been studied (Marquez *et al.* 1997; Pol *et al.* 2001), with the results indicating that *Bacillus* spores are damaged by an electrical field potential of ≥35 kV/cm. The mechanism of spore inactivation could be explained by the pulse polarity, whereby many ions within the cortex act as a shielding layer, so that instead of the electrical field penetrating the core, the cortex is destroyed by ionic interfacial polarization. However, the exact mechanism has not been established. Hayakawa *et al.* (1998) reported a method of inactivating the heat-tolerant *Bacillus stearothermophilus* IFO 12550

spores by rapid decompression. Those authors found that heat-tolerant spores such as those of *B. stearothermophilus* could not be sterilized using simple pressurization methods at pressures below 400 MPa. Pressurizing and heating spores at 200 MPa and 75C for 60 min killed 10^4 colony-forming units (CFU)/mL, with the sterilization being due to the physical breakdown of the spore coat that was induced by its physical permeability to water at high pressure and temperature.

The combination of physical methods employing variable energy levels is more effective than using a single energy to inactivate spores. Inactivation of *B. subtilis* spores by ultrasonic waves under pressure (manosonication, MS) and by a combined mild heat/MS treatment has been evaluated. The combination of heating to 70–90C with MS treatment (with the following parameters: 20 kHz, 300 kPa, 117 μm and 6 min) had a synergistic effect on spore inactivation. These results suggest that the mechanical effects of ultrasound are responsible for the damage and loss of viability of *B. subtilis* spores (Raso *et al.* 1998; Chandler *et al.* 2001).

The combination of various physical preservation methods with antimicrobial agents may also have a synergistic effect on spore inactivation. The fruits of *Torilis japonica* DC (Umbelliferae) have been used as an anti-inflammatory traditional medicine to cure skin diseases, arthroneuralgia and urogenital disorders (Lee and Ryu 1978; Kitajima *et al.* 1998). Our previous studies to screen novel antimicrobial substances against *B. subtilis* spores from 79 types of medicinal plants presented that the ethanol extract of *T. japonica* fruit showed excellent antimicrobial activity of about 3-log reduction against the spores (Cho *et al.* 2007, 2008). Torilin, a guaiane-type sesquiterpenoid angelate, is known as a major constituent of the fruit, and its various bioactivities that include anti-inflammatory and anticancer activities have been demonstrated (Kitajima *et al.* 1998). The present study investigated the synergistic sporicidal effects of the ethanol extract from *T. japonica* fruit on inactivation of *B. subtilis* spores induced by UV irradiation at a wavelength of 254 nm (UV_{254}) and elucidated the underlying mechanism.

MATERIALS AND METHODS

Ethanol Extract of *T. japonica* Fruit

Dried *T. japonica* fruits were purchased at a local market in Seoul, Korea, and stored at 4C until used. The dried *T. japonica* fruits were washed to remove extraneous substances and then redried for 3 h at 40C. Then the fruits ground into a fine powder, 400 g samples of which were then dispersed in 75% ethanol (2 × 4 L at 4 L/day) for 2 days at room temperature with shaking. The solvent was refreshed every day; and the extracted solvents were combined, filtered through Whatman no. 1 paper (Whatman, Maidstone, UK) and concentrated by vacuum evaporation (EYELA, Tokyo, Japan) at 40C (Choi *et al.* 2002). The concentrated extracts were lyophilized at 40C for 48 h.

The antimicrobial activity of ethanol extract from *T. japonica* fruit against spores and vegetable cells of *B. subtilis* was measured by colony counting on tryptic soy broth (TSB; Difco, Detroit, MI) agar plate as follows: The extracts were added at various final concentrations (0–2.0%) to the 1-mL aliquot of the spore suspension containing 1×10^6 spores in sterile 0.85% NaCl solution and 0.1% of Tween 80. The tube was incubated for 3 h at 30C, and then the suspensions were washed three times by repeated centrifugation/resuspension with 1 mL of sterile 0.85% NaCl solution to avoid any effects of the extract residue. The final spore pellet was resuspended in 1 mL of sterile 0.85% NaCl solution. Each spore suspension was inoculated to 5 mL of TSB medium and incubated at 37C for 18 h. The number of viable spores was determined by the standard colony count method on TSB agar medium.

Spore Preparation

B. subtilis ATCC 6633 cells were incubated on nutrient agar at 30C for more than 1 week. The vegetative cells with endospores were suspended in sterile 0.85% NaCl solution and then sonicated for 5 min (with a 15:15 s on : off cycle) to destroy vegetable cells and obtain the spores. A pellet containing endospores was collected by centrifugation at 4,200× g for 20 min at 4C. The cell pellet was washed and suspended in sterile 0.85% NaCl solution, and the spore condition was examined using modified Wirtz-Conklin spore stain (Schaeffer and MacDonald 1933; Hamouda *et al.* 2002). A 1-mL aliquot of the spore suspension containing 1×10^6 spores was stored in a 1.5 mL plastic cryopreservation tube at −70C until use (Chaibi *et al.* 1997).

UV Irradiation

A 1-mL aliquot of spore suspension of *B. subtilis* cells (1×10^8 cfu/mL) was mixed with 99 mL of sterile, distilled water and then 0.2 mL of the spore suspension (1×10^6 cfu/mL) was spread onto a 1.5% agar plate. Each spore-laden agar plate was treated by UV light of various wavelengths (185, 254 and 306 nm) with/without ethanol extract of *T. japonica* fruit at concentration of 1.0%, and then tryptic soy broth (Difco) medium including 0.75% agar was poured onto the agar plate in order to germinate the spore to vegetative cell. The number of spore was determined by counting vegetative cells after 24 h of incubation at 37C for spore germination (longer incubations led to negligible increases in the number of CFU). Experiments were performed in triplicate. The experimental UV sterilization

chamber with a continuous conveyer comprised a stainless-steel case equipped with 185 nm (UV_{185}), 254 nm (UV_{254}), 306 nm (UV_{306}) and 360 nm (UV_{360}) UV lamps.

RESULTS AND DISCUSSION

Sporicidal Effect of UV Irradiation

The sporicidal effect of UV irradiation on *B. subtilis* spores was investigated at different wavelengths (185, 254 and 306 nm) for treatment of 0–10 min. As shown in Fig. 1A, UV_{254} exhibited the strongest inactivation effect, with the level of spores being reduced by approximately 3.6-log cycles after 3 min of exposure. Exposure to UV_{185} caused a 2.5-log reduction in *B. subtilis* spores after 3 min. The degree of spore kill achieved with UV_{254} and UV_{185} was further increased to about 4.7-log reduction by increasing the exposure time to 10 min. Also, the inactivation of *B. subtilis* spores treated with UV_{254} was examined at irradiation distance (10–30 cm) for exposure time of 10 min (Fig. 1B). At an irradiation distance of 10 and 15 cm, UV_{254} treatment produced 5.0- and 3.6-log reduction, respectively, in the number of spores after 3 min of exposure. The viability of the spores decreased with increasing exposure time and when the irradiation distance was reduced from 30 to 10 cm. The extent of the inactivation was essentially independent of the irradiation distance at exposure times of >10 min. On the basis of the above results, we evaluated the UV_{254} dose–response behavior of *B. subtilis* spores on agar plates (Fig. 1C). The energy dose of radiation was analyzed using the D_{10} value ($\mu W \cdot min/cm^2$), which is the energy required to inactivate 90% of the original number of spores. The inactivation of spores was found to depend upon the applied dose, with the dose–response curve exhibiting biphasic inactivation kinetics, comprising a rapid kill at low UV_{254} doses followed by a slow kill at high doses.

It has been documented that UV irradiation is effective at killing bacteria that contaminate the surfaces of various materials. It has also been established that the inactivation of cells by UV irradiation is attributable primarily to its lethal effects on DNA. The purine and pyrimidine bases of nucleic acids absorb UV radiation strongly, and the maximum absorption for DNA and RNA occurs at a wavelength of 254 nm. Exposure to UV radiation can induce several other deleterious effects, such as abnormal ion flow, increased cell membrane permeability and depolarization of the cell membrane (Slieman and Nicholson 2000; Slieman *et al.* 2000; Lee *et al.* 2001; Reidmiller *et al.* 2003).

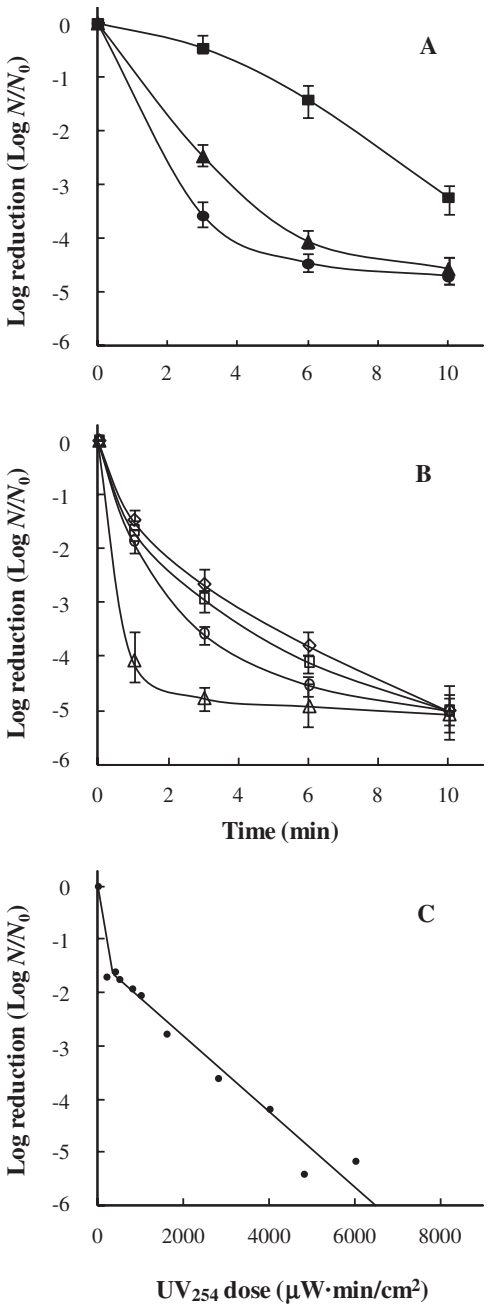

FIG. 1. (A) INACTIVATION OF *B. SUBTILIS* SPORES EXPOSED TO UV RADIATION AT DIFFERENT WAVELENGTHS (▲: 185 NM, •: 254 NM, ■: 306 NM). (B) INFLUENCE OF IRRADIATION DISTANCE (△: 10 CM, ○: 15 CM, □: 20 CM, ◊: 30 CM) AND EXPOSURE TIME ON THE LETHAL EFFECT OF UV_{254} TREATMENTS. (C) UV_{254} DOSE–RESPONSE BEHAVIOR OF THE SPORES. EXPERIMENTS WERE PERFORMED IN TRIPLICATE. DATA ARE MEAN AND STANDARD DEVIATION VALUES

Sporicidal Effect of Ethanol Extracts of *T. japonica* Fruit

The sporicidal effect of ethanol extract of *T. japonica* fruit was evaluated as a function of concentration of the extract (0–2.0%) for 0–3 h. As shown in Fig. 2, the viability of *B. subtilis* spores decreased with increasing concentration and treatment time. In the early stages of the treatment (30 min), approximate 1.3-, 2.0- and 2.5-log reductions

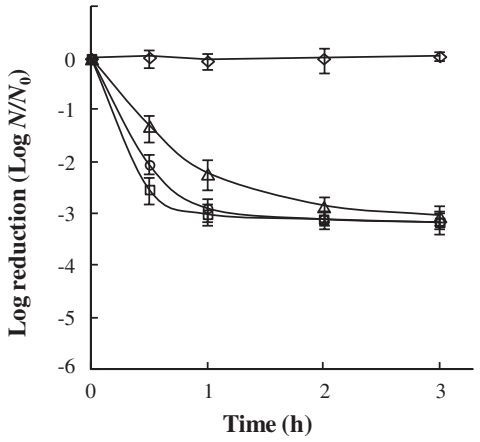

FIG. 2. INACTIVATION OF *B. SUBTILIS* SPORES EXPOSED TO ETHANOL EXTRACT OF *T. JAPONICA* FRUIT WITH DIFFERENT CONCENTRATIONS (◊: 0%, △: 0.5%, ○: 1.0%, □: 2.0%) FOR AN EXPOSURE TIME OF 3 H. EXPERIMENTS WERE PERFORMED IN TRIPLICATE. DATA ARE MEAN AND STANDARD DEVIATION VALUES

were observed at concentrations of 0.5%, 1.0% and 2.0%, respectively. However, spore reductions of about 3.0-log were obtained for the treatment time of 1 h at 1.0% and 2.0%, and also the treatment lasting 3 h produced 3.0-log reductions at all the concentrations.

T. japonica contains various antimicrobial components, such as β-pinene, β-pinene, lpinene, bornyl acetate, osthol, limonene, β-pinenelimonene, α-phellandrene, β-phellandrene, β-cymene, camphene, δ-3-carene, β-eudesmol, β-caryophyllene, α-cadinene, pinocarveol, γ-terpinene, geranyl acetate and dithiothreitol, that can kill and inhibit *Trichomonas* species, Newcastle disease virus and influenza (Lee *et al.* 1998). Moreover, previous studies have been reported that *T. japonica* fruit contains torilin, which is a guaiane-type sequiterpene compound, and germinants such as L-alanine and potassium (Cho *et al.* 2008, 2009). Therefore, the inactivation of *B. subtilis* spores by the ethanol extract of *T. japonica* fruit may occur in the following three stages: (1) direct inactivation of the spores by the surfactant function of torilin, (2) synergistic effects due to disruption of spore coat after initiation of germination and cortex hydrolysis by germinants such as L-alanine and potassium and (3) inactivation of vegetative cells (Cho *et al.* 2009).

Combined Treatment with UV Irradiation and Ethanol Extract of *T. japonica* Fruit

The present study investigated the inactivation of *B. subtilis* spores under concurrent UV_{254} irradiation and treatment with ethanol extract of *T. japonica* fruit in order to ascertain whether there was any synergistic sporicidal effect and to elucidate the sporicidal mechanism. The sporicidal mechanism of this combined treatment was evaluated by assessing the survival rates of *B. subtilis* spores under various spore conditions using UV_{254} irradiation with or without the addition of the ethanol extract of *T. japonica* fruit. Four spore conditions were assessed: (1) intact spores, (2) early-germination, decoated spores, (3) outgrowth spores with a hydrolyzed cortex and (4) vegetative cells. The strains for each growth condition were produced by incubating the spores on nutrition broth with germinants (Fig. 3).

As shown in Fig. 4A, UV_{254} irradiation alone exhibited 3.0–3.8-log reductions in spores at growth stages lower than vegetative cells for an exposure time of 3 min, and approximate 4.8-log reduction was observed in vegetative cells. The difference between the results for spores and vegetative cells can be explained by spore structure and chemical composition. Although it can be presumed that for the communication system, the inner coats would be connected to UV-resistant factors residing in the core, the actual resistance mechanisms against UV exposure remain to be established. The UV resistance of the spore is attributable mainly to the existence of a cortex in the dehydration state (as a

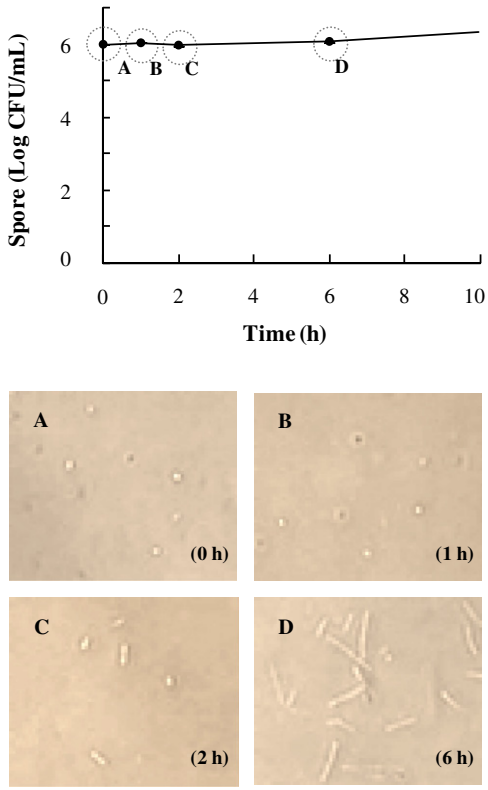

FIG. 3. CHANGES IN *B. SUBTILIS* SPORE CONDITION ACCORDING TO CULTIVATION TIME

(A) Intact spores (0 h). (B) Decoated spores on germination (1 h). (C) Cortex-hydrolyzed outgrowth spores (2 h). (D) Vegetative cells (6 h).

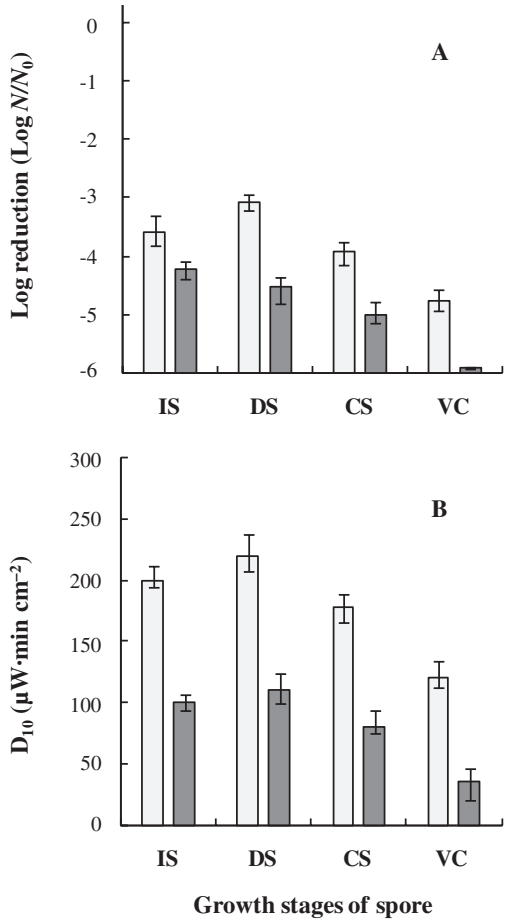

FIG. 4. (A) INACTIVATION EFFECTS AND (B) D_{10} VALUES OF COMBINED TREATMENT WITH UV_{254} IRRADIATION AND 1% ETHANOL EXTRACT OF *T. JAPONICA* FRUIT ON *B. SUBTILIS* SPORES AT DIFFERENT GROWTH STAGES FOR AN EXPOSURE TIME OF 3 MIN AND A TREATMENT DISTANCE OF 15 CM

IS, intact spores; DS, decoated spores on germination; CS, cortex-hydrolyzed outgrowth spores; VC, vegetative cells. □: UV_{254} irradiation alone; ■: combination treatment of UV_{254} irradiation and 1% ethanol extract of *T. japonica* fruit. D_{10} is the energy dose of radiation required to inactivate 90% of the microorganism (i.e., reduce to one-tenth). Experiments were performed in triplicate. Data are mean and standard deviation values.

result of the contractile cortex theory), acid-soluble DNA-binding protein and the dehydration condition of the core (Nicholson *et al.* 2000). However, in the early stage of germination, the spore survival was about 0.6-log cycles higher in decoated spores than in intact spores, despite the lack of a UV-resistant inner coat. Regardless of the inactivation of spores by UV irradiation, if UV_{254} promotes germination via a photocatalyst reaction (similar to the catalytic effects on germination of treatment with high-pressure and mechanical stress), it may initiate the rapid germination, outgrowth and growth of spores.

Furthermore, the present results show that combined treatment with UV irradiation and ethanol extract of *T. japonica* fruit produced 4.2-, 4.5- and 5.0-log reductions in intact, decoated and outgrowth spores, respectively, for an exposure time of 3 min; and approximate 6.0-log reduction was obtained in vegetative cells (Fig. 4A). These results indicate that the sporicidal effects in intact spores, decoated spores at the early-germination stage and outgrowth spores with a hydrolyzed cortex were 0.7-, 1.5- and 1.1-log greater reduction in spores compared with UV irradiation alone. In this study, we also evaluated the D_{10} value ($\mu W \cdot min/cm^2$) of UV_{254} with 1% ethanol extract of *T. japonica* fruit on *B. subtilis* spores at different growth stages, with an exposure time of 3 min and a treatment distance of 15 cm. The results of Fig. 4B indicate that, in spores of all the growth stages, the energy required from UV irradiation to inactivate the spores was about 50% lower in the presence of 1% ethanol extract of *T. japonica* fruit than with UV irradiation alone. Besides, the LD_{90} value, which is the sterilization time to inactivate 90% of microorganisms, on the inactivation of the intact spores shows about 50% decrease in the value, from 0.60 min at UV irradiation alone to 0.32 min at the combined treatment (Fig. 5).

The results presented herein demonstrate the synergistic sporicidal interaction between UV_{254} irradiation and 1% ethanol extract of *T. japonica* in the killing of *B. subtilis* spores, with a short exposure time of up to 3 min. As stated above, this synergistic effect on the inactivation of *B. subtilis*

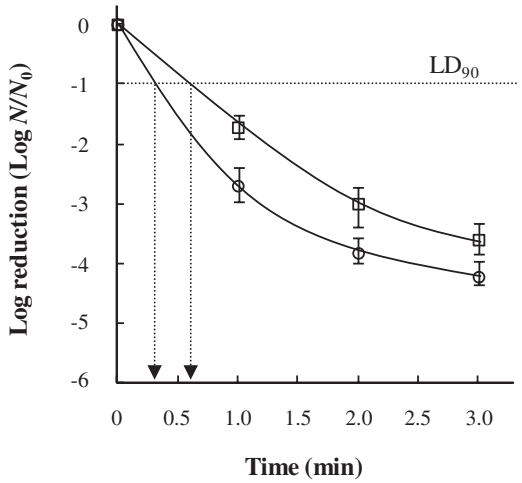

FIG. 5. COMPARISON BETWEEN LD_{90} VALUES OF UV_{254} IRRADIATION ALONE (□) AND COMBINED TREATMENT (○) OF UV_{254} WITH 1% ETHANOL EXTRACT OF *T. JAPONICA* FRUIT ON THE INTACT SPORES OF *B. SUBTILIS* FOR AN EXPOSURE TIME OF 3 MIN AND A TREATMENT DISTANCE OF 15 CM

LD_{90} is the sterilization time required to inactivate 90% of the microorganism. Experiments were performed in triplicate. Data are mean and standard deviation values.

spores might be attributable to the following three factors: (1) destruction of the spore cortex, the inner coat, and the communication system by UV-resistant factors residing in the core; (2) torilin, which is an antimicrobial component; and (3) L-alanine and potassium, which are germinants of the ethanol extract of *T. japonica* fruit. The surfactant properties of torilin enable it to induce decomposition of the inner coat, and the germinants, as a synergist, trigger hydrolysis of the cortex during germination and outgrowth of the spores (Riesenman and Nicholson 2000; Cho *et al.* 2009).

CONCLUSIONS

UV irradiation and natural antimicrobial components can act synergistically to kill bacteria: both vegetative cells and spores. Advantage has been taken of this synergistic action to design more effective sterilization procedures that are especially directed toward the killing of spores. Consequently, the findings in this study indicate that the sporicidal effect of UV irradiation on *B. subtilis* spores was further enhanced after brief exposure to the ethanol extract of *T. japonica* fruit; thus, the spores could be effectively killed by combined treatment with UV irradiation and ethanol extract of *T. japonica* fruit. The present findings suggest that the combined treatment of UV and *T. japonica* fruit extract can become a promising new method for the decontamination of *Bacillus* spores from food products.

ACKNOWLEDGMENTS

This research was supported by an RP-Grant 2011 from Ewha Womans University and by a grant (10162KFDA995) from Korea Food and Drug Administration in 2011.

REFERENCES

CHAIBI, A., ABABOUCH, L., BELASRI, K., BOUCETTA, S. and BUSTA, F.F. 1997. Inhibition of germination and vegetative growth of *Bacillus cereus* T and *Clostridium botulinum* 62A spores by essential oils. Food Microbiol. *14*, 161–174.

CHANDLER, D.P., BROWN, J., BRUCKNER-LEE, C.J., OLSON, L., POSAKONY, G.J., STULTS, J.R., VALENTINE, N.B. and BOND, L.J. 2001. Continuous spore disruption using radially focused, high-frequency ultrasound. Anal. Chem. *73*, 3784–3789.

CHO, W.I., CHOI, J.B., LEE, K., CHO, S.C., PARK, E.J., CHUNG, M.S. and PYUN, Y.R. 2007. Antimicrobial activity of medicinal plants against *Bacillus subtilis* spore. Food Sci. Biotechnol. *16*, 1072–1077.

CHO, W.I., CHOI, J.B., LEE, K., CHUNG, M.S. and PYUN, Y.R. 2008. Antimicrobial activity of torilin isolated from *Torilis japonica* fruit against *Bacillus subtilis*. J. Food Sci. *73*, M37–M46.

CHO, W.I., CHEIGH, C.I., CHOI, Y.J., JEONG, J.Y., CHOI, J.B., LEE, K., CHO, S.C., PYUN, Y.R. and CHUNG, M.S. 2009. Inactivation mechanism of *Bacillus subtilis* spores by ethanol extract of *Torilis japonica* fruit. Food Sci. Biotechnol. *18*, 336–342.

CHOI, I., CHANG, H.S., YUN, Y.M. and UM, J.C. 2002. Antimicrobial activity of medicinal herbs against *Staphylococcus aureus* and *Salmonella gallinarum*. Korean J. Microbiol. Biotechnol. *30*, 177–183.

HAMOUDA, T., SHIN, A.Y. and BAKER, J.R., Jr. 2002. A rapid staining technique for the detection of the initiation of germination of bacterial spores. Lett. Appl. Microbiol. *34*, 86–90.

HAYAKAWA, I., FURUKAWA, S., MIDZUNAGA, A., HORIUCHI, H., NAKASHIMA, T., FUJIO, Y., YANO, Y., ISHIKURA, T. and SASAKI, K. 1998. Mechanism of inactivation of heat-tolerant spores of *Bacillus stearothermophilus* IFO 12550 by rapid decompression. J. Food Sci. *63*, 371–374.

KITAJIMA, J., SUZUKI, N. and TANAKA, Y. 1998. New guaiane-type sesquiterpenoid glycosides from *Torillis japonica* fruit. Chem. Pharm. Bull. *46*, 1743–1747.

LEE, E.B., KIM, S.M. and KIM, T.H. 1998. Anti-inflammatory activities of *Torilis japonica* fruit. Korean J. Pharmacogn. *29*, 384–390.

LEE, I.H., PARK, J.S., KIM, S.W., CHA, J.M., CHOI, K.J. and LEE, B.G. 2001. Disinfection of coliform bacteria, fungi and pathogenic bacteria by UV and photocatalytic effects. J. Korean Soc. Environ. Eng. *23*, 473–483.

LEE, M.C. and RYU, K.S. 1978. Studies of the constituents in the fruits of *Torilis japonica* DC. Bull. KH Pharm. Sci. *6*, 61–67.

MARQUEZ, V.O., MITTAL, G.S. and GRIFFITHS, M.W. 1997. Destruction and inhibition of bacterial spores by high voltage pulsed electric field. J. Food Sci. *62*, 399–401.

NICHOLSON, W.L., MUNAKATA, N., HORNECK, G., MELOSH, H.J. and SETLOW, P. 2000. Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. *64*, 548–572.

POL, I.E., VAN ARENDONK, W.G.C., MASTWIJK, H.C., KROMMER, J., SMID, E.J. and MOEZELAAR, R. 2001. Sensitivities of germinating spores and carvacrol-adapted vegetative cells and spores of *Bacillus cereus* to nisin and pulsed-electric-field treatment. Appl. Environ. Microbiol. *67*, 1693–1699.

RASO, J., PALOP, A., PAGAN, R. and CONDON, S. 1998. Inactivation of *Bacillus subtilis* spores by combining ultrasonic waves under pressure and mild heat treatment. J. Appl. Microbiol. *85*, 849–854.

REIDMILLER, J.S., BALDECK, J.D., RUTHERFORD, G.C. and MARQUIS, R.E. 2003. Characterization of UV-peroxide killing of bacterial spores. J. Food Prot. *66*, 1233–1240.

RIESENMAN, P.J. and NICHOLSON, W.L. 2000. Role of spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl. Environ. Microbiol. *66*, 620–626.

SCHAEFFER, A.B. and MACDONALD, F. 1933. A simplified method of staining endospores. Science *77*, 194.

SHEARER, A.E., DUNNE, C.P., SIKES, A. and HOOVER, D.G. 2000. Bacterial spore inhibition and inactivation in foods by pressure, chemical preservatives, and mild heat. J. Food Prot. *63*, 1503–1510.

SLIEMAN, T.A. and NICHOLSON, W.L. 2000. Artificial and solar UV radiation induces strand breaks and cyclobutane pyrimidine dimmers in *Bacillus subtilis* spore DNA. Appl. Environ. Microbiol. *66*, 199–205.

SLIEMAN, T.A., REBEIL, R. and NICHOLSON, W.L. 2000. Spore photoproduct (SP) lyase from *Bacillus subtilis* specifically binds to and cleaves SP (5-thyminyl-5,6-dihydrothymine) but not cyclobutane pyrimidine dimers in UV-irradiated DNA. J. Bacteriol. *182*, 6412–6417.

SPILIMBERGO, S., ELVASSORE, N. and BERTUCCO, A. 2002. Microbial inactivation by high-pressure. J. Supercrit. Fluids *22*, 55–63.

WUYTACK, E.Y., BOVEN, S. and MICHIELS, C.W. 1998. Comparative study of pressure-induced germination of *Bacillus subtilis* spores at low and high pressures. Appl. Environ. Microbiol. *64*, 3220–3224.