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RESEARCH NOTE

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Antimicrobial Activity of Medicinal Plants Against *Bacillus subtilis* Spore

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Abstract Bacterial endospores, especially those of *Bacillus* and *Clostridium* genera, are the target of sterilization in various foods. We used *Bacillus subtilis* ATCC 6633 spores to screen novel antimicrobial substances against spores from medicinal plants. We collected 79 types of plant samples, comprising 42 types of herbs and spices and 37 types of medicinal plants used in traditional medicine in Korea and China. At a concentration of 1%(w/v), only 14 of the ethanol extracts exhibited antimicrobial activity against *B. subtilis* spores of at least 90%. Crude extracts of *Torilis japonica*, *Gardenia jasminoides*, *Plantago asiatica*, *Fritillaria*, and *Arctium lappa* showed particularly high sporicidal activities, reducing the spore count by about 99%. Consideration of several factors, including antimicrobial activity, extraction yields, and costs of raw materials, resulted in the selection of *T. japonica*, *G. jasminoides*, *A. lappa*, and *Coriandrum sativum* for the final screening of novel antimicrobial substances. Verification tests repeated 10 times over a 4-month period showed that the ethanol extract of *T. japonica* fruit reduced aerobic plate counts of *B. subtilis* spores the most, from 10^7 to 10^4 CFU/mL (99.9%) and with a standard deviation of 0.21%, indicating that this fruit is the most suitable for developing a novel antimicrobial substance for inactivating *B. subtilis* spores.

Keywords: *Bacillus subtilis*, endospore, sporicidal activity, medicinal plant, *Torilis japonica* fruit

Introduction

Bacteria of the *Bacillus* and *Clostridium* genera form stable dormant endospores that are more resistant than most vegetable cells to common sterilization and disinfection treatments, including heat, radiation, and chemicals (1-3). Many factors increase the resistance of a spore, including a thick proteinaceous coat, low water content, and high levels of dipicolinic acid and divalent cations in the spore core (1, 4). The spore coat provides the first line of defense against chemical treatments, with coats composed by two layers of protein being resistant to mechanical shocks (e.g., high pressures) and electric energy (5-9). The peptidoglycan cortex of the *Bacillus* spore represents an important barrier to the entry of small hydrophilic molecules into the central spore core (1, 5). Moreover, this cortex comprises a matrix structure of peptidoglycan, glycopeptide (spore mucopeptide), and Ca^{2+} that is resistant to heat and UV irradiation. The spore also has a dormant mode, in which it can endure conditions that are bad for growth. Therefore, special attention must be paid to the processing and preserving of foods so that spores are either inactivated or prevented from undergoing germination and outgrowth.

Heat treatment is the predominant method used to prevent spoilage and inactivate pathogens in foods. However, high-temperature sterilization is required to inactivate spores, and this can adversely affect the flavor, taste, and nutritive value of foods. Although some chemical food preservatives have a long history of safe use, few antimicrobial agents are able to inactivate spores, and even chemicals that are considered to the sporicidal must be used at much higher concentrations than those considered necessary for bactericidal effects (2, 10). The use of nonthermal processes and a combination of preservation methods have been suggested for food preservation, but their limited lethality against bacterial spores has discouraged attempts to use them as sterilizing procedures (7, 11, 12).

Antimicrobial components of plant origin are present in plant stems, roots, leaves, bark, flowers, and fruits (13). Indeed, plants may represent a poorly exploited source of antimicrobial agents because their structures and modes of action are not known. There are many medicinal plants in Korea that are potential sources of antimicrobial agents (14-16).

The objectives of this study were to measure the antimicrobial activity of herbs and medicinal plants and to screen novel antimicrobial substances against *Bacillus subtilis* spores.

Materials and Methods

Plant materials Dried medicinal plants were purchased at a local market at Seoul, Korea and stored at 4°C.

Spore suspension The ATCC 6633 strain of *B. subtilis* used as a test microorganism in this study was obtained from the culture collection of the Food and Bioprocess Engineering Laboratory of Yonsei University and incubated on nutrient agar at 30°C 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

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15:15-sec on:off cycle) so as to destroy the vegetable cells. Spores were collected by centrifugation with 12,000×g for 2 min and subsequently washed by repeated centrifugation/resuspension in sterile distilled water at 4°C. After 2 washes, spore pellets were resuspended in sterile 0.85% NaCl solution. The spore condition was examined using Dorner's method. Plastic cryopreservation tubes (1.5 mL) containing 1 mL of spore suspension (10^6-10^7 CFU/mL) were stored at -70°C until used (17).

The spore suspension was smeared on a glass slide and fixed with a Bunsen flame for microscopy observations. Slides were flooded with 5% aqueous malachite green (Fisher Scientific, Fair Lawn, NJ, USA) and then intermittently heated with a Bunsen flame over a period of approximately 5 min to ensure that the dye remained just below boiling point. Slides were rinsed with tap water, counterstained with 0.5% Safranin-O (Sigma Chemical, St. Louis, MO, USA) for 1 min, dried, and then examined under a light microscope (BH-2; Olympus, Tokyo, Japan) (18).

Table 1. Antimicrobial activities of herbs and spices against *B. subtilis* spores

Common name	Scientific name	Form	Moisture content (%)	Yield (%)	OD_{600}
Control (No added)	-	-	-	-	0.769
Basil	*Ocimum basilicum*	Powder	10.22	15.29	0.765
Basil leaves	*Ocimum basilicum*	Whole	10.74	14.10	1.060
Bay leaves	*Laurus nobilis*	Powder	8.55	22.61	0.530
Bay leaves	*Laurus nobilis*	Whole	9.08	21.46	0.444
Celery	*Apium graveolens*	Powder	10.21	27.09	0.121
Celery seed	*Apium graveolens*	Whole	10.45	13.18	0.825
Cinnamon	*Cinnamomum cassia*	Powder	12.04	26.88	0.868
Coriander	*Coriandrum sativum*	Powder	7.89	9.97	0.195
Coriander	*Coriandrum sativum*	Whole	6.54	9.06	0.063
Dill seed	*Anethum graveolens*	Powder	9.62	19.72	0.347
Dill seed	*Anethum graveolens*	Whole	10.84	6.59	0.425
Caraway	*Carum carvi*	Powder	9.90	19.96	0.000
Marjoram	*Origanum majorana*	Powder	9.57	20.53	0.261
Marjoram leaves	*Majorana hortensis*	Whole	10.84	20.37	0.007
Nutmeg	*Myristica fragrans*	Powder	10.92	14.24	0.394
Oregano leaves	*Origanum vulgare*	Whole	11.95	18.39	0.190
Rosemary	*Rosmarinus officinalis*	Whole	10.55	18.91	0.501
Sage	*Salvia officinalis*	Powder	11.16	14.50	0.033
Spearmint	*Mentha spicata*	Whole	8.56	7.75	0.684
Tarragon	*Artemisia dracunculus*	Powder	5.77	23.92	0.440
Thyme leaves	*Thymus vulgaris*	Whole	14.43	18.42	0.216
Allspice	*Pimenta dioica*	Whole	12.47	9.86	0.755
Cardamon seed	*Elettaria cardamomum*	Powder	15.15	5.49	0.441
Clove	*Eugenia caryophyllus*	Whole	13.22	23.33	0.445
Fennel seed	*Foeniculum vulgare*	Whole	6.75	8.22	0.513
Mace	*Myristica fragrans*	Whole	11.99	16.26	0.003
Star anise seed	*Illicium verum*	Whole	12.76	21.88	0.497
Turmeric	*Curcuma aromatica*	Whole	7.94	7.47	0.252
Nutmeg seed	*Myristica fragrans*	Whole	12.51	10.01	0.387
Garlic	*Allium sativum*	Flake	7.16	56.86	1.156
Onion	*Allium cepa*	Flake	15.58	73.62	0.551
White pepper	*Piper nigrum*	Whole	11.92	0.65	1.507
Black pepper	*Piper nigrum*	Whole	12.46	4.03	1.074
Mustard seed	*Brassica juncea*	Whole	7.31	12.69	0.821
Parsley	*Petroselinum crispum*	Flake	6.36	21.54	1.097
Schizandra berry	*Schizandra chinensis*	Whole	13.29	42.37	1.321
Satsuma mandarin	*Citrus unshiu*	Whole	12.78	29.19	1.081
Balloon flower	*Platycodon grandiflorum*	Whole	11.65	17.63	0.347
Milk-Vetch root	*Astragalus membranaceus*	Whole	8.67	14.27	0.065
Green tea	*Thea sinensis*	Whole	4.47	26.34	0.746
Chrysanthemum	*Chrysanthemum morifolium*	Whole	10.86	28.42	0.561

Measurements of antimicrobial activity The antimicrobial activity of medicinal plants extracts against *B. subtilis* spores was measured by optical density (OD) analysis and colony counting on tryptic soy broth agar (TSA) plates as follows. Five-hundred μL of 2% ethanol extract of each medicinal plant and 500 mL of spore suspension were mixed in a test tube and incubated in a shaker at 30°C for 2 hr. The suspensions were then washed 3 times by repeated centrifugation/resuspension with 1 mL of sterile 0.85% NaCl solution to avoid 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 37°C for 18 hr. The OD of the cell suspension was measured at 600 nm. Also, the number of viable spores was determined by a standard colony-counting method on TSA medium.

Extraction procedure Collected samples were washed to remove the extraneous materials and then ground into fine powders. Samples (400 g) of fine powders were dispersed in 75% ethanol (2×4 L) for 2 days at room temperature with shaking, with 4 L of 75% ethanol exchanged every day. The supernatant were combined, filtered through Whatman No. 1 paper and concentrated by rotary vacuum evaporation (Eyela, Tokyo, Japan) at 40°C. The concentrated extracts were lyophilized at 40°C for 48 hr, and the lyophilized extracts were dissolved in a small amount of 75% ethanol to produce a concentration of 2%. These extracts were used for screening antimicrobial substances.

Table 2. Antimicrobial activities of medicinal plants against *B. subtilis* spores

Common name	Scientific name	Part of plant used	Moisture content (%)	Yield (%)	OD_{600}
Control (No added)	-	-	-	-	0.7832
Forsythiae fruit	*Forsythia viridissima*	Seeds	11.24	13.92	0.8787
Angelicae gigantis radix	*Angelica gigas*	Roots	12.45	40.51	0.4792
Chaenomelis fructus	*Chaenomeles sinensis*	Fruits	14.68	31.01	0.5928
Akebiae caulis	*Akebia quinata*	Stems	9.77	9.46	0.6110
Cimicifugae rhizome	*Cimicifuga heracleifolia*	Roots	10.71	23.39	0.4120
Portulaca oleracea	*Portulaca grandiflora*		9.56	19.02	0.3943
Lonicerae flos	*Lonicera japonica*	Flowers	9.80	26.54	0.5046
Coptidis rhizome	*Coptis japonica*		11.47	20.87	0.4946
Mentae herba	*Mentha arvensis*	Leaves	11.45	18.52	0.7301
Artemisiae capillaris herbs	*Artemisia capillaries*	Leaves	12.30	9.85	0.4798
Houttuyniae herba	*Houttuynia cordata*	Leaves	12.26	8,01	0.3939
Dianthi herba	*Dianthus chinensis*	Leaves	13.84	10.95	0.3888
Patrinia	*Patrinaia villosa*	Stems	8.29	15.09	0.6407
Torilidis fructus	*Torilis japonica*	Fruits	12.62	6.60	0.3521
Taraxaci herba	*Taraxacum platycarpum*	Leaves	12.24	13.63	0.3435
Prunellae spica	*Prunella vulgaris*	Leaves	12.10	14.44	0.5359
Stemona japonica	*Stemona japonica*		10.34	60.36	0.4341
Saussureae radix	*Saussurea lappa*	Roots	11.64	21.65	0.4793
Trichosanthis semen	*Trichosanthes kirilowii*	Seeds	7.63	5.71	0.7129
Sophorae flavescentis radix	*Sophora flavescens*	Roots	10.12	17.61	0.2377
Arminiaceae semen	*Prunus armeniaca*	Seeds	3.17	5.29	0.4381
Artemisiae argyi herba	*Artemisia asiatica*	Leaves	13.94	8.05	1.1629
Acori graminei rhizome	*Acorus gramineus*	Roots	13.23	7.91	0.6305
Persimmon leaves	*Diospyros*	Leaves	9.99	18.69	0.4601
Phellodendri cortex	*Phellodendron amutense*	Barks	10.52	13.35	0.6812
Assam indigo powder	*Assam indigofera*	Powder	2.37	5.67	0.6238
Gardeniae fructus	*Gardenia jasminoides*	Fruits	8.86	22.93	0.3050
Achyranthis radix	*Achyranthes japonica*	Roots	11.52	31.69	0.7618
Fritillary	*Fritillaria*	Roots	15.06	6.70	0.3068
Plantaginis semen	*Plantago asiatica*	Seeds	8.99	1.23	0.2050
Honeysuckle	*Lonicera japonica*	Stems	11.06	9.31	0.7004
Cinnamomi ramulus	*Cinnamomum cassia*	Barks	18.26	4.24	0.4894
Arctii semen	*Arctium lappa*	Seeds	7.10	16.63	0.2462
Cirsii japonici herba	*Cirsium japonicum*	Roots	10.98	15.08	0.4094
Chinese anemone	*Chinese anemone*	Roots	10.53	29.00	0.8640
Coicis semen	*Coix lacryma-jobi*	Seeds	6.65	4.02	0.2544
Scrophulariae radix	*Scrophularia vuergeriana*	Roots	11.02	51.06	0.8955

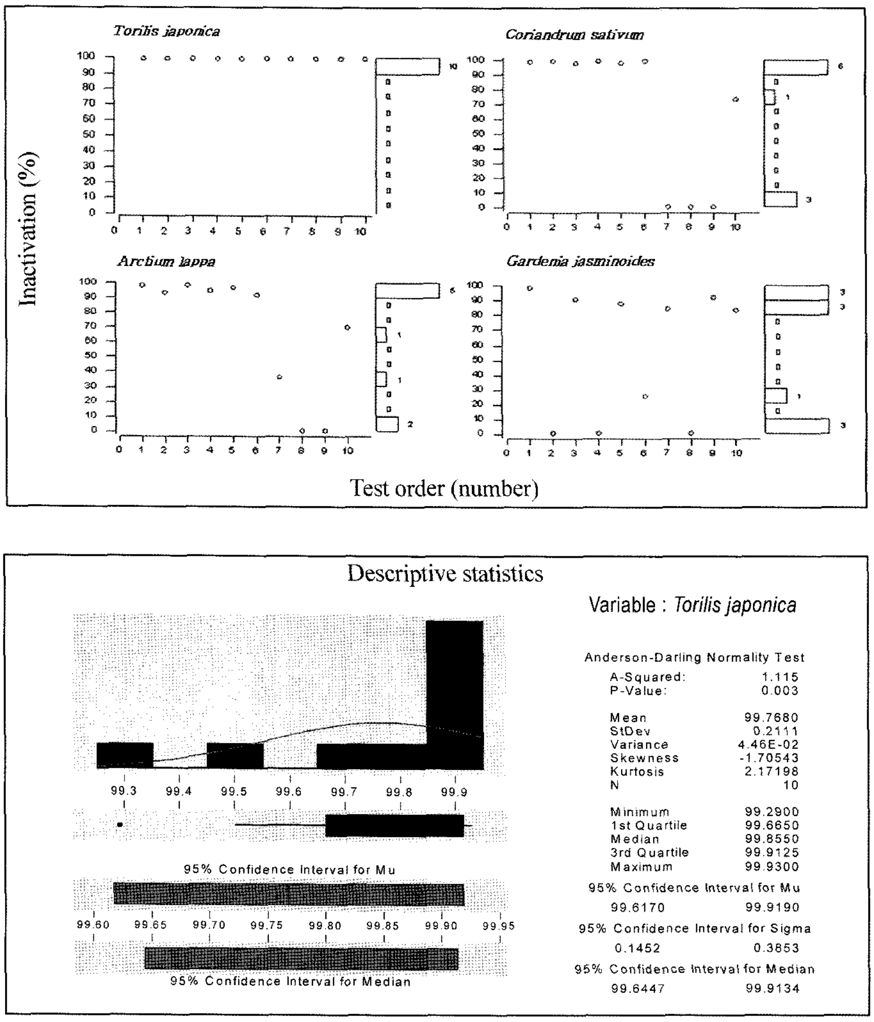
Results and Discussion

Antimicrobial effects of materials against *B. subtilis* spore We collected 79 types of plant samples known to exert natural antibacterial effects, comprising 42 types of herbs and spices and 37 types of medicinal plants used in traditional medicine in Korea and China. The antimicrobial activities of the herbs, spices, and medicinal-plant ethanol extracts are listed in Table 1 and 2. Most of the plant ethanol extracts exerted inactivation effects against *B. subtilis* spores, of which 23 that showed low ODs (<0.4) were selected for the second screening of the viability of spores performed by a plate-count method. At a concentration of 1%(w/v), only 14 of the ethanol extracts exhibited antimicrobial activity against *B. subtilis* spore of at least 90%. Crude extracts of *Torilis japonica*, *Gardenia jasminoides*, *Plantago asiatica*, *Fritillaria*, and *Arctium lappa* showed particularly high sporicidal activities, reducing the spore count by about 99% (Table 3).

Antimicrobial activity of selected materials against *B. subtilis* spores Consideration of several factors, including antimicrobial activity, extraction yield, and costs of raw

Table 3. Inactivation of *B. subtilis* spores by ethanol extracts of selected plants

Scientific name	Yield (%)	*B. subtilis* spores (CFU/mL)	Inactivation efficacy (%)
Control	-	6.96×10^4	-
Torilis japonica	6.6	5.00×10^2	99.282
Gardenia jasminoides	22.93	7.50×10^2	98.922
Plantago asiatica	1.23	7.50×10^2	98.922
Fritillaria	6.70	9.00×10^2	98.707
Arctium lappa	16.63	9.50×10^2	98.635
Taraxacum platycarpum	13.63	1.60×10^3	97.701
Coriandrum sativum	9.97	1.83×10^3	97.366
Sophora flavescens	17.61	2.05×10^3	97.055
Platycodon grandiflorum	17.63	2.14×10^3	96.931
Coix lacryma-jobi	4.02	2.55×10^3	96.336
Carum carvi	19.96	2.79×10^3	95.992
Dianthus chinensis	1.95	2.85×10^3	95.905
Astragalus membranaceus	14.27	3.19×10^3	95.420
Houttuynia cordata	8.01	3.35×10^3	95.187



Variable : *Torilis japonica*

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Anderson-Darling Normality Test
   A-Squared:          1.115
   P-Value:            0.003

   Mean              99.7680
   StDev              0.2111
   Variance         4.46E-02
   Skewness         -1.70543
   Kurtosis          2.17198
   N                      10

   Minimum           99.2900
   1st Quartile      99.6650
   Median            99.8550
   3rd Quartile      99.9125
   Maximum           99.9300
 95% Confidence Interval for Mu
   99.6170           99.9190
95% Confidence Interval for Sigma
    0.1452            0.3853
95% Confidence Interval for Median
   99.6447           99.9134
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Fig. 1. Comparison of antimicrobial activities of 4 medicinal plants against *B. subtilis* spores and descriptive statistics of sporicidal activity of *T. japonica* fruit.

materials, resulted in the selection of *T. japonica*, *G. jasminoides*, *A. lappa*, and *Coriandrum sativum* for the final screening of novel antimicrobial substances. Verification tests repeated 10 times over a 4-month period showed that the antimicrobial activity against *B. subtilis* spores was higher and more consistent for *T. japonica* than for any other plants (Fig. 1). The ethanol extract of *T. japonica* fruit reduced aerobic plate counts of *B. subtilis* spores from 10^7 to 10^4 CFU/mL (99.9%) with a standard deviation of 0.21% (Fig. 1). This contrasts with *G. jasminoides*, *A. lappa*, and *C. sativum*, which exhibited very high standard deviations ranging from 41.9 to 45.3%. Therefore, *T. japonica* fruit was finally selected as the most suitable for developing a novel antimicrobial substance for inactivating *B. subtilis* spores.

Sporicidal activity of *T. japonica* fruit against *B. subtilis* spore *T. japonica Decandolle* grows spontaneously nationwide in Korea as a 60-cm-high straight stem. The fruits of this plant (Korean name '*sasangja*') have been used traditionally for curing skin diseases since ancient times in Korea and China, and also in the treatment of testitis, arthroneuralgia, impotence, and inflammation (19). A recent study revealed that the hexane fraction of *T. japonica* exerts anti-inflammatory and analgesic effects, and that the acetone precipitates of the hot water extract of *T. japonica* showed strong hemostatic activity that was not inhibited by aspirin (20, 21).

The chemical constituents of *T. japonica* fruit include many sesquiterpenoids such as torilin, essential oils, and hemiterpenoids (21-23). Torilin reportedly exhibits anti-inflammatory, anticancer, and anti-invasive activities in human fibrosarcoma cells (21-23). Studies that have isolated components from *T. japonica* have revealed various components, including cadinene, torilene ($C_{22}H_{32}O_5$, sesquiterpene acetate), guaian, torilolide ($C_{20}H_{28}O_4$), oxytorilolide ($C_{20}H_{28}O_5$), α-thujene, α-pinene, camphene, β-pinene, δ-3-carene, limonene, α-phellandrene, β-phellandrene, γ-terpinene, bornyl acetate, carotol, β-cymene, β-caryophyllene, geranyl acetate, β-methoxy benzyl acetate, and 7-heptadeca-1,9-dien-4,6-diyn-3-ol (20). *T. japonica* also contains various antimicrobial compo-nents, such as β-pinene, β-pinene, l-pinene, 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 (20).

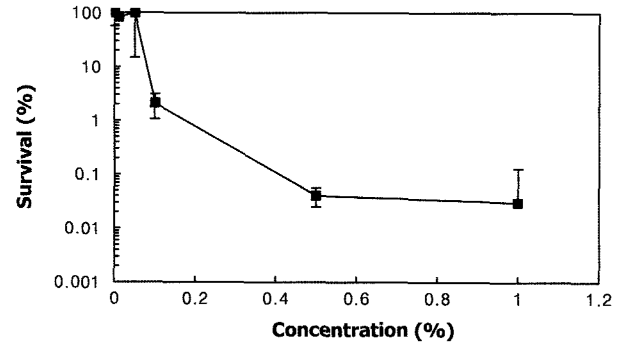

Fig. 2. Effect of concentration of ethanol extract of *T. japonica* on antimicrobial activity against *B. subtilis* spores. Mean and SD values are shown.

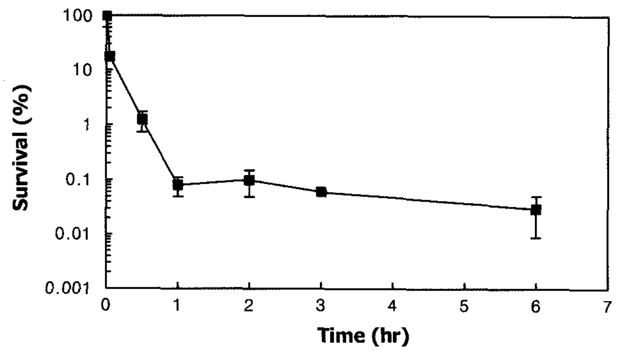

Fig. 3. Effect of incubation time of ethanol extract of *T. japonica* fruit (at a concentration of 1%) on antimicrobial activity against *B. subtilis* spores. Mean and SD values are shown.

We found that the inactivation of ethanol extract of *T. japonica* fruit at concentrations of 0.1 and 0.5-1.0% were about 100- and 1,000-fold (reductions of 99 and 99.9%), respectively. The minimum concentration at which inhibition occurred was estimated to be 0.1%(w/v) (Fig. 2). The level of *B. subtilis* spores was reduced 100-fold after 30 min and 1,000-fold after 60 min, and thereafter remained at a constant level (Fig. 3).

We are currently using various fractionation, isolation, and purification methods to assess the antimicrobial components and killing mechanism of *T. japonica* fruits against *B. subtilis* spores.

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