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Antimicrobial Activities of 1,4-Benzoquinones and Wheat Germ Extract

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We evaluated the antibacterial activities of selected edible Korean plant seeds against the food-borne pathogens *Staphylococcus aureus* KCTC1927, *Escherichia coli* KCTC2593, *Salmonella typhimurium* KCTC2054, and *Bacillus cereus* KCTC1014. While screening for antibacterial agents, we discovered that wheat germ extract contains 2,6-dimethoxy-1,4-benzoquinone (DMBQ) and is highly inhibitory to *S. aureus* and *B. cereus*. This is the first report of the antibacterial activity of wheat germ extract. We also investigated the antibacterial activities of the 1,4-benzoquinone standards 1,4-benzoquinone (BQ), hydroquinone (HQ), methoxybenzoquinone (MBQ), and 2,6-dimethoxy-1,4-benzoquinone (DMBQ). DMBQ and BQ were the most highly inhibitory to *S. aureus* and *S. typhimurium*, followed by MBQ and HQ. MICs for DMBQ and BQ ranged between 8 and 64 μg/ml against the four foodborne pathogens tested. DMBQ and BQ showed significant antibacterial activity; the most sensitive organism was *S. aureus* with an MIC of 8 μg/ml. BQ exhibited good activity against *S. typhimurium* (32 μg/ml) and *B. cereus* (32 μg/ml). The results suggest that wheat germ extract has potential for the development of natural antimicrobials and food preservatives for controlling foodborne pathogens.

Keywords: Antibacterials, benzoquinone, foodborne pathogens, MIC, wheat germ

Natural antimicrobials have been developed to cure bacterial foodborne illnesses and to control the increasing bacterial resistance to antibiotics currently used in therapeutics [11, 14, 15, 18, 27, 28]. As such, antimicrobial research with natural products represents a new field. In addition, there is an increased need for natural preservatives in food, beverages, cosmetics, and food packaging.

More than 2,000 naturally occurring quinones, such as anthraquinones, naphthoquinones, and benzoquinones, are now known and have been found to be widely distributed in nature as intermediates in cellular respiration and photosynthesis [2, 4, 20]. Hydro- and benzoquinones are members of the naturally occurring quinones, which have an interesting biological mode of action. Most quinones, including ubiquinones and menaquinones, are often involved in electron transport [16]. They provide a role in defense as a result of their effectiveness in inhibiting the growth of bacteria, fungi, and parasites [8]; therefore, a number of them have various physiological activities as antimicrobial and anticancer compounds [21]. Specifically, some benzoquinones such as 2,6-dimethoxy-1,4-benzoquinone (DMBQ) exhibit cytotoxic effects in Ehrlich ascites tumor cells (EATC) [22, 23], and thereby inhibit tumor propagation. DMBQ derived from different plant species is of special interest. Recent studies have reported that DMBQs from the roots of *Gunnera perpensa* [5] and *Eucalyptus* tar [13] are strongly antibacterial. Furthermore, it has been shown that benzoquinone-producing plants possess specific therapeutic properties that may be responsible for their beneficial effect on human health [10].

In our preliminary screening of several plant seeds previously characterized as antibacterial agents against Gram-negative and -positive bacteria, we discovered that wheat germ extract (WGE) from *Triticum vulgaris* contains DMBQ and is highly inhibitory to *Staphylococcus aureus* KCTC1927 and *Bacillus cereus* KCTC1014. Wheat germ is the nutrient-rich embryo of the wheat kernel that is removed during the processing of whole wheat grains to white flour. It makes up about 2% to 3% percent of the entire wheat kernel. The antibacterial activity of wheat germ has rarely been reported.

We report herein the content of a major bioactive compound DMBQ in WGE by high-performance liquid

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chromatography (HPLC). We also evaluate the antibacterial activity of the standard benzoquinone derivatives 1,4-benzoquinone (BQ), hydroquinone (HQ), methoxybenzoquinone (MBQ), and DMBQ, using the well-diffusion assay against standard strains of *S. aureus*, *Escherichia coli, Salmonella typhimurium* KCTC2054, and *B. cereus*. To evaluate the relationship between molecular structure and antibacterial activity, we determined the minimum inhibitory concentration (MIC) and time-kill curve of the active compounds.

Materials and Methods

Materials

Wheat germ was purchased from DongA One Corporation (Seoul, Korea). BQ (98% purity), HQ (97%), MBQ (98%), DMBQ (97%), and chloramphenicol (CM, 98%) standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The test strains, *S. aureus* KCTC1927, *E. coli* KCTC2593, *S. typhimurium* KCTC2054, and *B. cereus* KCTC1014, were purchased from the Korean Collection for Type Cultures (Biological Resource Center, Daejeon, Korea). Nutrient broth (0.03% beef extract, 0.05% peptone) for the antibacterial assay was obtained from BBL Microbiology System (Cockeysville, MD, USA). Beef extract and peptone were purchased from Difco Laboratories (Detroit, MI, USA). Unless otherwise noted, all chemicals were purchased from Sigma.

Sample Preparation

Ground wheat germ (10 g) was dissolved in 250 ml of double distilled water and then extracted three times by shaking with 100 ml of chloroform ($CHCl_3$). The $CHCl_3$ layers were pooled, washed twice with distilled water, and dried over anhydrous Na_2SO_4. The filtrate was evaporated to dryness in a vacuum evaporator at a maximum temperature of 40°C. The dry material (600 mg) was finally redissolved in $CHCl_3$ and filtered through a 0.45-μm PTFE filter aid. Twenty μl of the final filtrate was injected into the HPLC column.

Antibacterial Assay

Antibacterial activity was detected by the modified disk diffusion method [1]. *S. aureus*, *E. coli, S. typhimurium*, and *B. cereus* were subcultured in nutrient medium and incubated for 18 h at 37°C (30°C for *B. cereus*), and then the bacterial cells were suspended in saline solution according to the McFarland protocol to produce a suspension of about 10^5 CFU/ml. A volume of 0.5 ml of this suspension was mixed with 150 ml of nutrient agar at 40°C and poured onto an agar plate (23×23 cm) in a laminar flow cabinet. Finally, agar wells were cut from the seeded agar medium using a hollow tube (7 mm diameter) and applying slight negative pressure to remove the plug of agar. Each test compound was dissolved in $CHCl_3$, and 150 μl was added to wells containing bacterial cells. Wells containing CM (100, 500, and 1,000 ppm) and $CHCl_3$ only were used as positive and negative controls, respectively. The susceptibility of the bacteria to the test compounds was determined by the formation of an inhibition zone after 18 h of incubation at 37°C. Experiments were run in triplicate, and the results are presented as mean values of the three measurements. The MIC was evaluated by the macrodilution test using the method of Christoph *et al.* [3] with slight modifications. Briefly, serial 2-fold dilutions of the test compounds were prepared in dimethylsulfoxide, and 0.5 ml of each dilution was added to 9.0 ml of nutrient broth. These solutions were inoculated with 0.5 ml of an overnight culture of *S. aureus*, *E. coli, S. typhimurium,* or *B. cereus*. After incubation of the cultures at 37°C (30°C for *B. cereus*) for 48 h, the MIC was determined as the lowest concentration of the test compound that demonstrated no visible growth.

HPLC Analysis of 1,4-Benzoquinones

The 1,4-benzoquinones in WGE were analyzed by an HPLC method [29] with minor modifications. The extract was prepared as described above. The HPLC system consisted of a Tosoh 8010 series (Tosoh Corporation, Japan) equipped with a UV 8010 diode-array UV-vis detector (Tosoh) at 275 nm, and an RP-Amide C16 (250×4.6 mm) column (Supelco, Bellefonte, PA, USA). The mobile phase used a water:acetonitrile [80:20 (v/v)] mixture containing 0.0025 M KH_2PO_4, where the flow rate and sample injection volume were fixed at 0.7 ml/min and 20 μl, respectively. As the reference ingredient, DMBQ in 100% $CHCl_3$ was used to calibrate the standard curve and retention times.

Time-Kill Study

Bacteria were cultivated with each compound as described above for the determination of MIC. At selected time points, samples were withdrawn and serially diluted in sterile saline, plated on nutrient agar. The plates were incubated at 37°C for 48 h, and then the number of colony-forming units (CFU) was determined.

Statistical Analysis

All data are presented as means ± SD. Statistical analyses were carried out using the Statistical Package for Social Science (SPSS; SPSS Inc., Chicago, IL, USA).

Table 1. Antibacterial activities of wheat germ extract, chloramphenicol (CM; positive control), and chloroform ($CHCl_3$; negative control) as measured by the inhibition zone test. (Units, mm)

	S. typhimurium	*E. coli*	*S. aureus*	*B. cereus*
Wheat germ extract[a]	-	-	27.63±1.7	17.8±0.7
$CHCl_3$	-	-	-	-
CM (100 ppm)	10.5±0.2	10.1±0.3	10.1±0.2	11.3±0.1
CM (500 ppm)	16.5±0.4	14.7±0.2	19.7±0.7	14.8±0.3
CM (1000 ppm)	18.3±0.2	17.7±0.4	24.2±1.4	17.3±0.7

[a]30 mg/150 μl.
The results (in mm) represent the means±SD of values obtained from three measurements.

RESULTS AND DISCUSSION

Antibacterial Activity and HPLC Analysis of Wheat Germ Extract

The antibacterial activities of WGE and the reference drug CM, as indicated by the zone of inhibition of Gram-positive bacteria (*S. aureus* and *B. cereus*) and Gram-negative bacteria (*E. coli* and *S. typhimurium*), are summarized in Table 1. WGE (30 mg of dry extract/150 μl) was strongly inhibitory of *S. aureus* (27.6 mm) and *B. cereus* (17.8), a result that is relevant to the potential management of food poisoning.

In order to identify the antimicrobial compounds in WGE, a chromatographic analysis was performed. WGE contained 108.7 μg of the major compound DMBQ per gram of dry extract. Significant amounts of the other benzoquinone derivatives BQ, MBQ, and HQ (Fig. 1) were not detected by this method. This result indicates that DMBQ may contribute to the antibacterial activity of WGE, and is in agreement with a previous study in which synthesized BQ and DMBQ exhibited antibacterial activity against *S. aureus* [13]. It is also consistent with the anti-*B. cereus* activity of benzoquinone derivatives from *G. perpensa* reported by Drewes *et al.* [5].

Thus, the chloroform extract of wheat germ, which has a high DMBQ content, has potential as a food-based source for the safe production and enhancement of antimicrobial components.

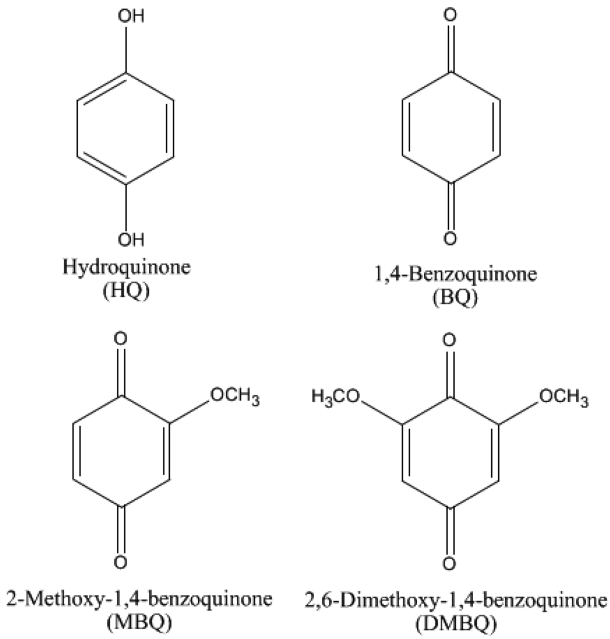

Fig. 1. Chemical structures of the benzoquinone compounds used in this study.

Antibacterial Spectrum of 1,4-Benzoquinones

Since WGE showed strong antibacterial activity against the selected foodborne pathogens (Table 1) and had a high DMBQ content, we compared its activity with that of various concentrations (100, 500, 1,000 ppm) of the 1,4-benzoquinone standards BQ, MBQ, DMBQ, and HQ (Table 2). Pure DMBQ had the highest anti-*S. aureus* activity (27.7 mm) followed by CM (24.2 mm), MBQ (14.2 mm), HQ (12.6 mm), and BQ (11.4 mm). These results reveal an interesting structure-dependent activity for these compounds. Among them, BQ is a 1,4-benzoquinone, whereas MBQ and DMBQ are methoxylated 1,4-benzoquinones. BQ was active against all four bacteria tested, whereas DMBQ and MBQ showed especially potent antibacterial activity against the Gram-positive bacterium *S. aureus* with

Table 2. Antibacterial activities of selected compounds against Gram-positive bacteria (*S. aureus* and *B. cereus*) and Gram-positive bacteria (*E. coli* and *S. typhimurium*). (Units, mm)

	μg/ml (ppm)	*S. typhimurium*	*E. coli*	*S. aureus*	*B. cereus*
BQ	100	-	-	-	-
	500	11.1±0.2	10.4±0.1	-	11.8±0.2
	1,000	13.4±0.1	11.4±0.1	11.4±0.2	12.3±0.1
MBQ	100	-	-	-	-
	500	-	-	12.2±0.4	12.4±0.6
	1,000	10.4±0.2	10.1±0.2	14.2±0.7	13.9±0.7
DMBQ	100	-	-	19.9±0.5	11.6±0.2
	500	19.6±0.2	-	26.6±1.3	16.6±0.7
	1,000	23.5±0.3	-	27.7±0.7	19.2±0.3
HQ	100	-	-	-	-
	500	-	-	10.8±0.1	-
	1,000	-	-	12.6±1.1	-
CM	100	10.5±0.2	10.1±0.3	10.1±0.2	11.3±0.1
	500	16.5±0.4	14.7±0.2	19.7±0.7	14.8±0.3
	1,000	18.3±0.2	17.7±0.4	24.2±1.4	17.3±0.7

The results (mm) represent the mean±SD of values obtained from three measurements.

an inhibition zone of 27.7 and 14.2 mm in diameter, respectively. In the case of DMBQ, the anti-*S. aureus* (27.7 mm) and anti-*S. typhimurium* (23.5) activities were higher than that of the reference antibiotic CM (24.2 and 18.3 mm, respectively) at a concentration of 1,000 ppm.

Many of the benzoquinone derivatives tested showed a comparable and selective inhibition of the foodborne pathogens. These results suggest that DMBQ, the major benzoquinone component of WGE, may play an important role in the antibacterial activity of wheat germ.

MICs of 1,4-Benzoquinones

The MICs of the active 1,4-benzoquinones were determined and are shown in Table 3. BQ was active against *S. aureus* up to a dose of 8 μg/ml, the same MIC found for DMBQ. Interestingly, the MICs observed for DMBQ, which bears two methoxy groups, were as high as those obtained for BQ, except when tested against *B. cereus*, in which case the value was twice as high.

This result is due to an interesting effect of the methoxy identity (presence of methoxy groups) of 1,4-benzoquinones. As shown in Table 3, the anti-*S. typhimurium* activity (MIC) of MBQ was significantly lower than that of DMBQ (>512 vs. 32 μg/ml, respectively). The difference in the number of methoxy groups in the 1,4-benzoquinones significantly affected their antibacterial activities against the Gram-negative bacteria *S. typhimurium* and *E. coli*; the introduction of one methoxy group in the nucleus resulted in decreased activity. Furthermore, HQ, a reduced form of BQ, had significantly lower antibacterial activity than BQ (Table 3). This result also indicates that reduction of BQ could decrease its antibacterial activity.

These data clearly demonstrate that the number of methoxy groups and reduction of BQ may play an important role in antibacterial activity, an observation that is in agreement with the previous study by Lana *et al.* [13]. Similar results have been reported by Koyama [10], who demonstrated that the dimethoxylated structure is crucial for potent antibacterial activity.

Time-Kill Curves of Selected 1,4-Benzoquinones

The bactericidal activities of BQ against *S. typhimurium* and *S. aureus* were confirmed by the time-kill curve experiment, as shown in Fig. 2 and 3.

Table 3. Minimal inhibitory concentration (MIC, in μg/ml) of hydroquinone and 1,4-benzoquinone standards.

Samples	*S. typhimurium*	*E. coli*	*S. aureus*	*B. cereus*
BQ	32	64	8	32
MBQ	>512	>512	16	32
DMBQ	32	32	8	64
HQ	512	256	64	>512

The result verifies that the MIC and minimal bactericidal concentration (MBC) of BQ against *S. typhimurium* were the same. The MIC of BQ (32 μg/ml) significantly reduced the growth rate of *S. typhimurium* (Fig. 2A). It should be noted that lethality occurred quickly, within the first hour after the addition of BQ, suggesting that the antibacterial activity of BQ against *S. typhimurium* was associated with membrane disruption, similar to its effect on *S. aureus* (Fig. 3A). The bactericidal effect of DMBQ was also confirmed by the time-kill curve experiment, as shown in Fig. 2B and 3B. The effect of DMBQ against *S. typhimurium* cells was bacteriostatic for the first 5 h of incubation after addition of the compound, but its bactericidal effect was expressed after 12 h of incubation. Lethality against *S. aureus* occurred more slowly than with BQ, 12 h after adding DMBQ (Fig. 3A and 3B). Thus, doubling the MIC of DMBQ to 16 μg/ml reduced the growth rate of *S. aureus* but did not have a siginificant effect on the final cell count (Fig. 3B). These results indicate that the modes

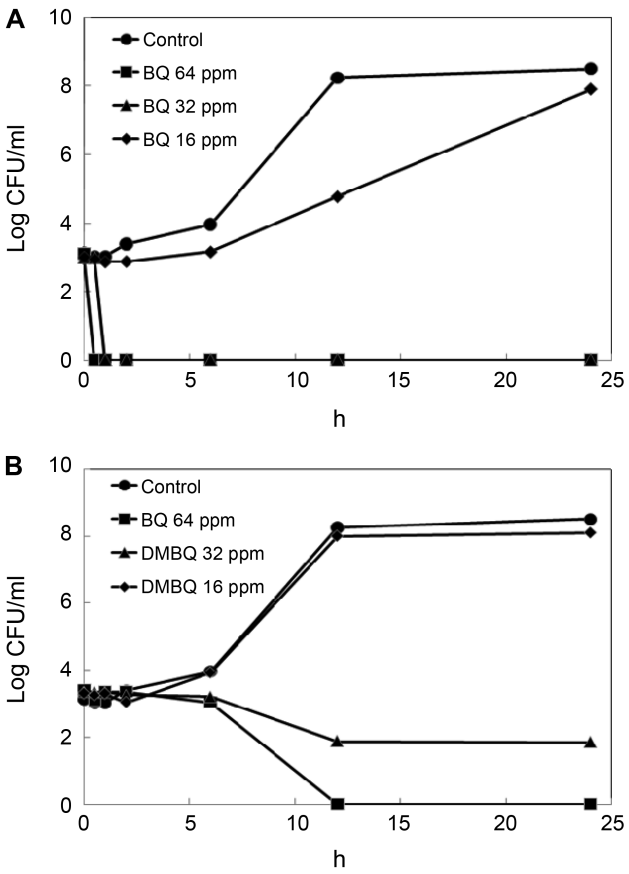

Fig. 2. Bactericidal effects of DMBQ and BQ against *S. typhimurium*.

Exponentially growing cells were inoculated into nutrient broth containing 0 (●), 16 (◆), 32 (▲), or 64 (■) μg/ml (ppm) of BQ (**A**) or DMBQ (**B**) at 37°C. Viability was determined by counting the number of colonies formed on nutrient agar plate after incubation at 37°C for 24 h.

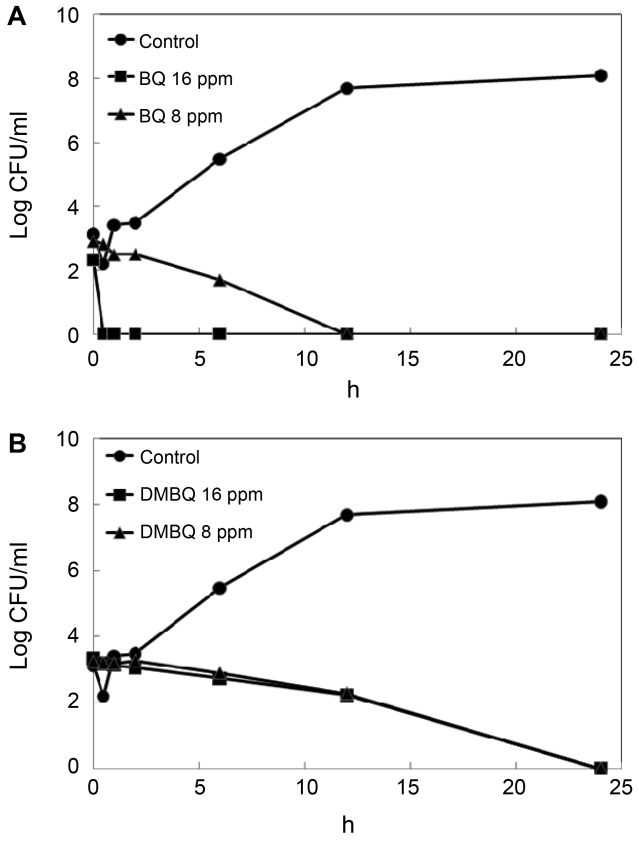

Fig. 3. Bactericidal effects of DMBQ and BQ against *S. aureus*. Exponentially growing cells were inoculated into nutrient broth containing 0 (●), 8 (▲), or 16 (■) μg/ml (ppm) of BQ (**A**) or DMBQ (**B**) at 37°C. Viability was determined by counting the number of colonies formed on nutrient agar plates after incubation at 37°C for 24 h.

of antibacterial action of BQ and DMBQ against *S. typhimurium* and *S. aureus* differ to some extent. Previous researchers have demonstrated that BQ is a potent sulfhydryl arylator that is highly cytotoxic [26]. BQ may have undergone arylation, binding to glutathione or protein thiols [26]. In contrast, DMBQ, while capable of strongly inducing oxidative stress by redox cycling, is only slightly cytotoxic [6].

Quinones are important biological molecules that are active against a variety of cancer cells [2, 25], viruses [12], and fungi [17]. Some semisynthetic analogs of substituted 1,4-benzoquinones have *in vitro* cytotoxic and antioxidant activities [19]. Quinones also play a critical role in energy metabolism and even in chemotherapy where redox cycling drugs are utilized. However, the molecular mechanisms involved in quinone cytotoxicity are still mostly unknown [26]. Since quinones are widely used as antibiotics and antitumor agents and for a variety of other purposes, it is critical that we understand their effects on cellular function.

The present study revealed the selective antibacterial activity of 1,4-benzoquinone derivatives against microorganisms that cause food poisoning [7, 9, 24] and once again points out the importance of structure in the antibacterial activity of these compounds. The antibacterial mechanism of 1,4-benzoquinone compounds from wheat germ against Gram-positive bacteria seems to be an interesting subject for further studies. The simple preparation of active 1,4-benzoquinone derivatives from wheat germ, reported herein, should be of interest to food industries, where such economically useful compounds could be extracted from waste materials. The toxicity of benzoquinone derivatives and structurally similar compounds is not well defined, but seems to be low enough to permit the development of new antimicrobials for human use or as agents to prevent bacterial food spoilage [13]. The results of the present study provide further insight into the molecular basis of the antibacterial action of these compounds, and a foundation for further studies regarding the production and enhancement of bioactive antimicrobial compounds using grains with high DMBQ content.

In conclusion, WGE could serve as a safe and inexpensive natural antimicrobial against foodborne pathogens for use in food and cosmetic applications. Wheat germ, a by-product generated in large quantities by the flour milling industry, could thus be utilized as a source of this antimicrobial agent.

Acknowledgments

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