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Antibacterial Activities of Polyphenolic Extract from Kiwi Fruit (*Actinidia chinensis* Planch.) Seeds

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Polyphenol extracted from kiwi fruit seeds (KSPE) were screened for their antimicrobial activity against six pathogenic bacterium. It had significant effects against all of the bacterial tested except E. coli. Among these bacterial strains, it showed a better antibacterial activities against Gram-positive than Gram-negative bacteria. Furthermore, based on MIC/MBC value, KSPE were identified as a bactericidal against *B. cereus, B. subtilis, S. flexneri* and *S. typhi* and bacteriostatic against *B. thuringiensis*. Growth curve results of *B. subtilis* also showed a significant decrease in turbidity and delay on growth log phase of the liquid cultured bacteria, indicating a potential as a food antibacterial ingredient of KSPE and provide a basis theory for food industries.

Key words: Kiwi fruit seed; polyphenolic; antibacterial activity.

As far as food safety is concerned, microorganisms have undesired effects on quality, safety and shelf life of foods in food industries. For example, pathogenic bacteria and viruses are all possible contaminants of foods. In order to inhibit the growth of spoilage and pathogenic microorganisms, many synthetic and artificial additives were used. However, there are a significant number that have recently been found to pose health risks after many years of use. Currently, seeking the new natural sources of the antimicrobial compounds has increased the interest.

Phenolic compounds, as the most numerous group of natural bioactive compounds, which are common found in fruit, vegetables and cereals, are contributed to many medicinaland food industries as natural materials (Biglari *et al.,* 2008; Gong [1]*et al.,* 2012; Busani *et al.,*2012). They have been reported to have a variety of biological effects, including anti-inflammatory, antimicrobial, anticarcinogenic, etc. (Cai *et al.,* 2004; Scalbert *et al.,* 2005). Specifically some phenolic compounds such as resveratrol,quercetin and a number of phenolicacidshave been reported to inhibit various pathogenicmicroorganisms.

Fruits and vegetables wastes and by-products, which are formed in great amounts during industrial processing, exert an influence on environment and need to be managed and utilized. Additionally, they are very rich in bioactive components from many kinds of fruit seeds, such as grape seeds (Cady *et al.,* 2010) and fenugreek seeds (Kannappan *et al.,* 2009). Kiwi fruit (*Actinidia chinensis* Planch.), also known as the Chinese gooseberry, is the edible berry which is originated from the central and southern regions

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of China. So far, the production of kiwi fruit in China is about 720 million kg, nearly half of the global yield. The kiwi fruit seeds, which represent about 33~46 g/kg of the edible parts of the kiwi fruit, were produced as solid wastes resulting from the food industries (Schieber, *et al.,* 2001). However there is limited research on the antimicrobial activities of polyphenol extract from kiwi fruit seed (KSPE), which are formed as wastes in great amounts during industrial processing.

The objectives of this work were therefore to investigate the antimicrobial activities of kiwi fruit seed polyphenols from solvent extractions and to determine the total phenolic compounds of the extracts to find out the relationship between antimicrobial activity and phenolic compound content.

MATERIALS AND METHODS

Materials

Defatted kiwi fruit seeds were obtained from Qinmei Co. (Xi'an, China). Grape seeds (Cabernet sauvignon) were supplied by Qinhuangdao Hwaseong Winery (Qinhuangdao, China). All samples werestored at -4 °C until used. All other reagents used were of analytical grade or purer. Distilled and deionized water was used throughout this study. The bacterial strains including *Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus, Escherichia coli, Shigella flexneri* and *Salmonella typhi* were obtained from the Nutrition and Food Safety Engineering Research Center, Shaanxi, China.

Polyphenol Extraction

The polyphenols from kiwi fruit seeds were extracted as previously described (Chedea, *et al.,* 2011). The harvested plant materials were dried in the laboratory at room temperature (20 ± 2 °C) and homogenized in four volumes of aqueous acetone (60%, v/v) using a Waring blender. The polyphenol extraction was carried out in a glass vessel by keeping it in a water bath (HH-S2, Zhengji, China) with 40 °C. The solution was continuously stirred with a magnetic stirrer for 30 min. The slurry was left 30 min at 4 °C and then centrifuged at 12, 000 × g for 30 min (Auanti J-26XP, Beckman, USA) to separate the insoluble materials. The supernatant was collected and the same procedure was used one more time to increase the polyphenol extractability. As control, grape seed polyphenol extract (GSPE) was also prepared by the method above at the optimum conditions. All the experiments were carried out in triplicate.

Total Phenolic Content (TPC) Assay

The TPC was determined by Folin-Ciocalteu colorimetric method (Singleton *et al.,* 1965). Estimations were carried out in triplicate and calculated from a calibration curve obtained with gallic acid. TPC were expressed as gallic acid equivalents (mg GAE/g DW).

Inoculum preparation

A loopful of isolated colonies was inoculated into 4 mL saline solution (0.85%, w/v)and incubated at 37°C for 12 h. The turbidity of actively growing bacterial suspension was adjusted to match the turbidity standard of 0.5 McFarland units prepared by mixing 0.5 mL of 150 g/L barium chloride dehydrate with 99.5 mL of 0.8 mmol/L sulphuricacid. This turbidity was equivalent to approximately 1×10^8 colony forming units per milliliter (cfu/mL). The suspension was used for further testing.

Antimicrobial bioassay

The antibacterial tests were performed by the Mueller Hinton (MH) agarwell diffusion method (Pal *et al.,* 2007). 15 mL of MH agar medium was dispensed into pre-sterilized 90 mm diameter Petri dishes to yield a uniform depth of 4 mm. The bacterial suspension was used to inoculate with a sterile non-toxic cotton swab on a wooden applicator. Wells (06 mm) were punched in the agar and filled with 200 µL polyphenol samples. Ampicillin sodium (AS) was used as positive controls and 70% actone was used as a negative control. The discs were spaced farenough to avoid reflections wave from the edges of the petridishes and overlapping rings of inhibition. Plates were incubated in air at 37°C for 24 h. All inhibitory tests were performed in triplicate. Antibacterial activities were evaluated by measuring inhibition zone diameters (mean of triplicates ± SD), which was devoid of growth of microbes.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

MIC was determined using the MH broth microdilution in 96 well-plates. The same 0.5 Mac Farland suspensions were diluted with MH broth to inoculate 96 well-plates containing 2-fold serial

dilutions of extracts. Drug concentrations ranged from 10 to 400 µg GAE/mL. The final volume in wells was 200 µL. Plates were incubated in air at 37°C for 24 h. MIC was recorded as lowest extract concentration demonstrating no visible growth in the broth. MBC was recorded as a lowest extract concentration killing 99.9% of bacterial inoculate. MBC values were determined by removing 100 µL of bacterial suspension from subculture demonstrating no visible growth and inoculating MH agar plates. Plates were incubated at 37°C for 24 h.

Evaluation of bactericidal and bacteriostatic capacity

The action of an antibacterial on the bacterial strains can be characterized with previous methods (Berche, *et al.,* 1998). If the ratio MBC/MIC = 1 or 2, the effect was considered as bactericidal but if the ratio MBC/MIC = 4 or 16, the effect was defined as bacteriostatic.

Growth inhibition assay (turbidometry and spectrophotometry)

A growth curve was plotted according to Farouk, et al. (Farouk *et al.,*2007) method with some modifications. Ten microliters of logarithmic-phase bacterial cultures (10^8 cfu/mL) in 200 µL nutrient broth was added in each well. KSPE with final concentrations from 0 to 40 µg GAE/mL medium, were cocultivated with *B. subtilis*. The cultures were incubated at 37°C for 24 h and growth inhibition was measured by determination of the absorbance at 545 nm.

Statistical analysis

The data were analyzed using the Statistical Analysis System (SAS 9.0) package software for analysis of variance, Duncan's test. All experiments were carried out in triplicate. The significance was established at $p \leq 0.05$.

RESULTS AND DISCUSSION

Polyphenol extraction

TPC in KSPE were determined using gallic acid as a standard. The amounts of TPC extracted with 70% acetone solvent at 40 °C were 49.52 ± 2.37mg GAE/g DW. Compared with polyphenol content extracted from grape seeds (101.76 ± 3.92 GAE/g DW), the content of KSPE gave a lower level. However, KSPE showed a higher content than polyphenol extracted from boragoofficinalisseeds (Mhamdi, *et al.,* 2010).

Antimicrobial activities

In this study, six bacteria strains including three Gram positive bacteria (*B. cereus, B.thuringiensis* and *B.subtilis*) and three Gram negative bacteria (*E. coli, S. flexneri* and *S. typhi*) were selected for estimate the antimicrobial activities of KSPE. The assay was performed by the agar-well diffusion so that they could be quantified by inhibition zone diameters, shown in Figure 1. It is shown that the susceptibility of the bacteria to the polyphenol from kiwi fruit seeds on the basis of inhibition zone diameters were significant different. As positive control, AS also has the high activities and 70% acetone, as negative control, has no inhibition effect in these bacterial tested. From Figure 1A, at the higher concentrations (600 mg GAE/g DW), the KSPE had significant differences followed the sequence: *B. subtilis* > *B. cereus* > *B. thuringiensis* > *S. flexneri* > *S. typhi* > *E. coli*. Among these strains, there is nearly no effect of KSPE on *E. coli*, which showed similar results with negative control. Compared with GSPE, KSPE showed a relative high activity on five bacterial stains except on *E. coli*. To testify the antibacterial effect, the inhibition test at low concentration (300 mg GAE/g DW) of polyphenol was also determined (Figure 1B). It is showed the similar results with the effect of polyphenol with high concentration above. These bacteria were important water and food contaminates in the world. The increasing level of the KSPE concentrations generally showed an increase in the inhibition effects and a reduction in the rate of growth of the test bacteria. It can be concluded that the KSPE in low concentrations may be useful as antibacterial additives to prevent the deterioration of stored foods by bacteria. Polyphenols have been reported to exhibit antibacterial activities with distinguished characteristics in their reactivity with proteins related polyamides polymers (Haslam, 1996). Additionally, KSPE showed relatively the better inhibitory activity against Gram-positive bacterium (*B. subtilis, B. cereus* and *B. thuringiensis*) followed by Gram-negative bacterium (*S. flexneri,S. typhi* and *E. coli*). The significance differences may be due to the different cell wall structures. The permeability of the cell wall of the Gram-negative organism is generally less efficient than Gram-positive ones probably because of the

presence of the high level of phospholipids in the cell wall compared with Gram-positive bacteria (Konaté, *et al.,* 2012).

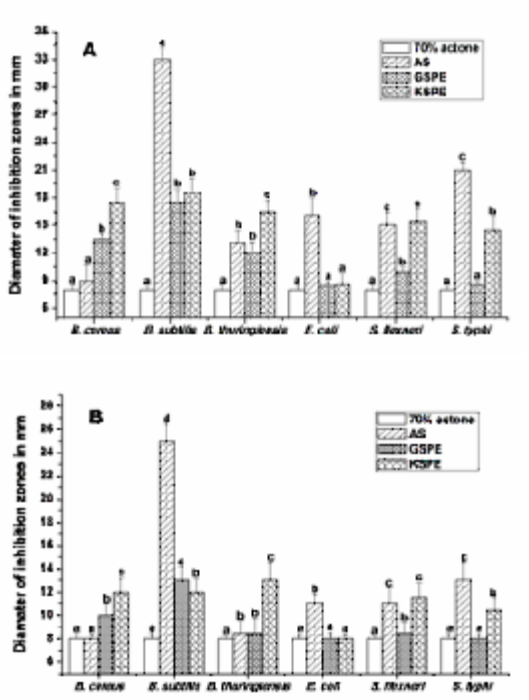

Fig. 1. Antimicrobial activities of KSPE against pathogenic B. cereus, B. subtilis, B. thuringiensis, E. coli, S. flexneri, and S. typhi. (A) [KSPE] = 600 µg GAE/mL, [GSPE] = 600 µg GAE/mL, [AS] = 100 µg/mL; (B) [KSPE] = 300 µg GAE/mL, [GSPE] = 300 µg GAE/mL, [AS] = 50 µg/mL. Bars with the same letters represent values that are not significantly different according to the least significance difference test ($P < 0.05$). Vertical bars represent the standard error

MIC and MBCof KSPE

Since the antibacterial activities of KSPE were quite significant different on these bacterial stains, MIC and MBC were determined in optimal activity conditions and the results varied according to the microorganisms, suggesting a selective activity of the KSPE (Table 1). Because of no effect of KSPE against *E. coli*, the experiments were carried out only on the other five strains. All the MIC values were 40 µg GAE/mL and for the MBC values were ranged from 40 to 320 µg GAE/mL. In order to elucidate whether the observed antimicrobial effects were bacteribicide or microbiostatic, MBC/MIC ratio were determined. From Table 1, it is shown that KSPE were bactericidal against *B. cereus*, *B. subtilis*, *S. flexneri* and *S. typhi* and bacteriostatic only against *B. thuringiensis*. Moyo, et al. (Moyo *et al.,*2012) reported that polyphenol from moringaoleifera Lam leaf had a bactericidal effect against *E. coli*, which is inconsistent with our results. It should be noted that for plant materials, there is actually no standard concentration as a model measure for determining the antibacterial activity.

Growth inhibitionresults

Measurements of the clear zone around the wells from the antibacterial activity tests gave a rough estimate of the efficacy of the KSPE and so the growth inhibition was evaluated by spectrometry measuring the turbidity of the liquid cultured bacteria. In this study, we determined the growth curve of one of Gram-positive bacterial *B. subtilis* as a typical example. Absorbance readings obtained from turbidometric analysis helped

Table 1. MIC and MBC of KSPE against pathogenic *B. cereus*, *B. subtilis*, *B. thuringiensis*,*S. flexneri*, and *S. typhi*.[a]

Tested bacterial strains	MIC (µg GAE/mL)	MBC (µg GAE/mL)	MBC /MIC	Effects
B. cereus	40.00 ± 2.14	80.00 ± 0.00	2	+
B.thuringiensis	40.00 ± 0.00	320.00 ± 0.00	4	-
B. subtilis	40.00 ± 0.00	80.00 ± 0.00	2	+
S. flexneri	40.00 ± 0.00	40.00 ± 0.00	1	+
S. typhi	40.00 ±1.12	80.00 ± 0.00	2	+

[a] values are the means ± standard deviations (n = 3).
+bactericidal effect, - bacteriostatic effect.

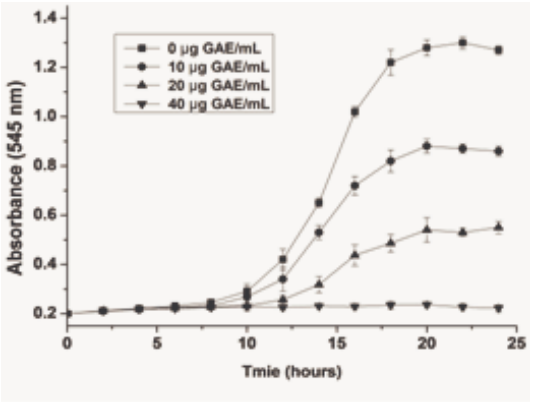

Fig. 2. Growth curves of *B. subtilis* measured by UV-Vis spectroscopy at 545 nm at different time intervals

determine the efficacy of the KSPE over time. Figure 2 showed the growth inhibition curves of *B. subtilis* treated by KSPE by UV-Vis spectroscopy at 545 nm at different time intervals. The reading was done up to 24 hours. As a negative control, the turbidity of the bacterial was strongly higher after 12 h. after 18 h of cultivation *B. subtilis* no longer increased and reached a platform. Compared with the sample treated by KSPE, the ODs of bacterial with different concentration of KSPE were lower than control, indicating that bacterial cell count at the lag phase was also lower. Moreover, with the KSPE concentration increase, the OD values decrease and the growth logarithmic phase was delayed. When the concentration was up to 40 μg GAE/mL, there was no bacterial growth within 24 h. Their antibacterial activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Deepa, *et al.,* 2012).

CONCLUSION

In short according our results, we demonstrated that KSPE could inhibit five bacterial strains except E coli. The polyphenol extracted from kiwi fruit seeds was found to possess promising antimicrobial activities. These results will aid in the search for new plant resources, which may be exploitable as an antibacterial additive to prevent the deterioration of stored foods by bacteria.

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