

# Isolation of Abscisic Acid from Korean Acacia Honey with Anti-*Helicobacter pylori* Activity

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## ABSTRACT

**Background:** *Helicobacter pylori* (*H. pylori*) is linked to the development of the majority of peptic ulcers and some types of gastric cancers, and its antibiotic resistance is currently found worldwide. **Objective:** This study is aimed at evaluating the anti-*H. pylori* activity of Korean acacia honey and isolating the related active components using organic solvents.

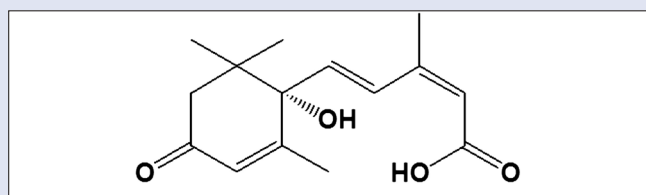
**Material and Methods:** The crude acacia honey was extracted with *n*-hexane, dichloromethane, ethyl acetate (EtOAc), and *n*-butanol. The EtOAc extract was subjected to octadecyl-silica chromatography. The extracts and fractions were then examined for anti-*H. pylori* activity using the agar well diffusion method. The antimicrobial activity of abscisic acid against *H. pylori* was investigated by determining the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), and by performing a time-kill assay. **Results:** Abscisic acid related to the botanical origins of acacia honey from Korea has been analyzed using ultra-performance liquid chromatography. The MICs and MBCs of abscisic acid were  $2.7 \pm 1.3$  and  $6.9 \pm 1.9$   $\mu\text{g/mL}$ , respectively. The bactericidal activity of abscisic acid (at 10.8  $\mu\text{g/mL}$  corresponding to  $4 \times \text{MIC}$ ) killed the organism within 36–72 h. These results suggest that abscisic acid isolated from Korean acacia honey has antibacterial activity against *H. pylori*. **Conclusion:** Abscisic acid isolated from Korean acacia honey can be therapeutic and may be further exploited as a potential lead candidate for the development of treatments for *H. pylori*-induced infections.

**Keywords:** Abscisic acid, acacia honey, antibacterial activity, *Helicobacter pylori*, UPLC

## SUMMARY

- The crude acacia honey was extracted with *n*-hexane, dichloromethane, EtOAc, and *n*-butanol

- The EtOAc extract yielded eight fractions and four subfractions were subsequently obtained chromatographically
- Abscisic acid was isolated from one subfraction
- All the solvent extracts and fractions showed antibacterial activity against *H. pylori*
- Abscisic acid exhibited antibacterial activity against *H. pylori*.



**Abbreviations used:** MeOH: Methanol; EtOAc: Ethyl acetate; TSB: Trypticase soy broth; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; CFU: Colony-forming units; UPLC: Ultra-performance liquid chromatography; DAD: Diode array detector; UV: Ultraviolet; ODS: Octadecyl-silica; MS: Mass spectrometry; SE: Standard error.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is considered as the causative agent of chronic gastritis, peptic ulceration, and gastric cancer.<sup>[1,2]</sup> Approximately 50 and 59.6% of the world and Korean populations, respectively, have been reported to be infected with *H. pylori*.<sup>[3,4]</sup> This Gram-negative, curved-rod bacterium colonizes the gastric epithelial surface and withstands the stomach's hostile acidic environment because of its microaerophilic growth capacity.<sup>[5]</sup> Treatment for the control of *H. pylori* infection has continued to advance from the standard triple therapy using a combination of agents such as antibiotics, bismuth subsalicylate, proton pump inhibitors, and H<sub>2</sub>-blockers.<sup>[6]</sup> However, eradication of the organism is incomplete and this treatment is not entirely curative and often associated with undesirable side effects. Therefore, nonantibiotic-based agents such as natural compounds, which are effective and free from side effects, may be beneficial for the control of *H. pylori*.<sup>[7]</sup>

Floral honey, a natural sweetener collected by bees from nectar, is a complex mixture of sucrose, fructose, glucose, water, proteins, amino acids, vitamins, minerals, and organic acids and widely used in numerous applications such as supplementary diets, tonics, and cosmetics.<sup>[8,9]</sup> It has

also been reported to have various biologic activities such as antioxidant, anti-inflammatory, and antibacterial.<sup>[10]</sup> Of course, the components of honey can vary depending on floral and geographical origins as well as seasonal and environmental climatic conditions.<sup>[11,12]</sup> It is generally known that honey has osmotic effects, is acidic, and contains hydrogen peroxide and plant-derived substances. Several kinds of floral honey exhibit additional antimicrobial activity, which has been attributed to various components including methylglyoxal, bee defensin-1, and other floral-derived phenolic compounds.<sup>[13,14]</sup> The aim of the present study

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was to evaluate the anti-*H. pylori* activity of acacia honey from Korea and isolate the active components mediating the antibacterial effect of the acacia honey.

## MATERIALS AND METHODS

### Acacia honey sample

The study was performed in 2014 and the honey samples used were the acacia (*Robinia pseudoacacia* L.) from Korea. This was purchased from 10 areas of the Korea Apicultural Agriculture Cooperative (Seoul, Korea) and certified by the cooperative and the Korea Food and Drug Administration.<sup>[15]</sup> The honey samples were stored in the dark and at 4°C before use.

### Instruments and reagents

The proton (1H-600 MHz) and carbon 13 (13C-150 MHz) nuclear magnetic resonance spectra were obtained using a JNM-ECA-600 spectrometer (JEOL, Japan). The chemical shifts were expressed in parts per million (ppm) relative to tetra-methylsilane as an internal standard and coupling constants (J) were expressed in Hertz (Hz). The mass spectrometry (MS) was performed using a liquid chromatography MS-ion trap-time-of-flight (LCMS-IT-TOF) system (Shimadzu, Japan). The column chromatography was performed using silica gel (Kieselgel 60, 40–63 µm, Merck, Germany), octadecyl-silica (ODS)-A resins (75-µm, YMC, Japan) and thin-layer chromatography (Merck, Germany). All other chemicals and reagents were analytical grade.

### Assay extraction and isolation

The crude acacia honey (10 kg) was extracted thrice with methanol (MeOH) with stirring and the solvent was evaporated *in vacuo* to obtain the MeOH residue (5.4 kg). The residue was suspended in water (H<sub>2</sub>O) and partitioned with *n*-hexane (3.5 g), dichloromethane (7.0 g), ethyl acetate (EtOAc, 9.0 g), and *n*-butanol (2.5 g). The EtOAc extract was further subjected to ODS column chromatography (5 cm × 80 cm) using a gradient of MeOH-H<sub>2</sub>O (1:4-4:1) to yield eight fractions (Fr. A1–A8). Fr. A5 was chromatographed on a silica gel column (1.5 cm × 50 cm) with CH<sub>2</sub>Cl<sub>2</sub>:MeOH:H<sub>2</sub>O (90:15:10) to yield three subfractions (Fr. 51-54). All fractions were monitored by susceptibility testing against *H. pylori*. Compound I (11mg) was separated using a recrystallization method from Fr. 54, and was analyzed by Waters (MN, USA) ultra-performance liquid chromatography (UPLC) I class system equipped with a BEH C18 (2.0 × 50 mm, 1.7 µm) column. The diode array detector (DAD) wavelength was set at 260 nm based on the UV absorption of compound I. This had the following characteristics. Compound I: white powder; ESI-MS *m/z* 264.1 [M+H]<sup>+</sup>; 1H-NMR (600 MHz, DMSO-*d*<sub>6</sub>): δ 7.73 (1H, d, *J* = 15.8 Hz, H-4), 6.22 (1H, d, *J* = 15.8 Hz, H-5), 5.81 (1H, s, H-3'), 5.67 (1H, s, H-2), 2.53 (1H, d, *J* = 16.5 Hz, H-5'b), 2.11 (1H, d, *J* = 16.5 Hz, H-5'a), 1.97 (3H, s, H-6), 1.82 (3H, s, H-7'), 0.96 (3H, s, H-9'), 0.92 (3H, s, H-8'); 13C-NMR (150 MHz, DMSO-*d*<sub>6</sub>): δ 197.2 (C-4'), 167.0 (C-1), 163.1 (C-2') 148.8 (C-3), 137.1 (C-5), 127.3 (C-4), 125.9 (C-2), 118.7 (C-3'), 78.3 (C-1'), 49.3 (C-5'), 41.2 (C-6'), 24.1 (C-9'), 23.2 (C-8'), 20.8 (C-6), 18.8 (C-7').

### Anti-*H. pylori* susceptibility testing

The anti-*H. pylori* assay was performed using the American Type Culture Collection (ATCC) strain ATCC 43526 from the Korean Culture Center of Microorganisms (Seoul, Korea). The *H. pylori* was cultured for 3 days at 37°C in trypticase soy broth (TSB, BBL, USA) containing 5% horse serum (MB cell, Korea) in an anaerobe container system (BD, USA). An agar well diffusion assay was performed according to the method of Dastouri *et al.*<sup>[16]</sup> to assess the antimicrobial activity of the acacia honey fractions. Briefly, cultured *H. pylori* (100 µL, OD<sub>540</sub> = 0.5) was added

to warm nutrient agar, which was then poured into plates and allowed to set for at least 6 h. Wells were cut into the agar plates using a sterile cork borer in a regular grid pattern. The honey samples were tested for antibacterial activity at a concentration of 25%. After incubation for 3 days, digital caliper used to measure the diameter of the inhibition halo zone around the wells.

### Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of abscisic acid was determined using the broth microdilution method in 96-well microtiter plates.<sup>[17]</sup> The abscisic acid was dissolved in distilled water and then filtered through a membrane filter (0.2 µm pore size, Millipore, Billerica, MA, USA). Then, the *H. pylori* suspension (1 × 10<sup>6</sup> colony-forming units [CFUs]/mL) was incubated with twofold serial dilutions of abscisic acid prepared in TSB containing 5% horse serum under anaerobic conditions for 24 h. The MIC was determined as the lowest concentration of abscisic acid that inhibited the visible growth of the test organism (optically clear). The positive control used was amoxicillin (Sigma-Aldrich, St. Louis, MO, USA).

### Determination of minimum bactericidal concentration

The minimum bactericidal concentration (MBC) value was determined as the lowest concentration of abscisic acid required to achieve a 99.9% reduction in the viable *H. pylori* cell population (NCCLS, 2008). To determine the MBC values, an aliquot (0.1 mL) of each MIC mixture that showed no growth was inoculated onto trypticase soy agar plates and incubated at 37°C for 48 h. No trailing was observed.

### Time-kill assays

The time-kill assays were performed using previously described standard Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>[18]</sup> Briefly, bacterial suspensions were diluted with appropriate broth media to 1.8 × 10<sup>8</sup> CFUs/mL of *H. pylori*, pre-incubated at 37°C and the samples were co-incubated with abscisic acid adjusted to 1.0% in TSB to a final concentration of the MIC, 2 × MIC, and 4 × MIC. Then, 100 µL aliquots of the culture collected before (0 h, positive control) and 18 h after the addition of abscisic acid were used to estimate the CFUs on the appropriate agar plates with adequate dilutions using buffered saline supplemented with 0.01% gelatin. Three plates were used for each sample, the CFU estimation was repeated separately, and the plates were later incubated at 37°C for 72 h.

### Statistical analysis

Data are presented as mean ± standard error (SE). Experimental results were statistically analyzed using the Duncan's *t*-tests (SAS Enterprise Guide, SAS Institute Inc., Cary, NC, USA).

## RESULTS

### Anti-*H. pylori* activity of solvent extracts/fractions of acacia honey and abscisic acid identification

The crude acacia honey showed detectable antibacterial activity against the *H. pylori* strains with inhibition zone diameters of 9.2 ± 5.8 mm at a concentration of 20% (v/v) [Table 1]. All the solvent extracts and fractions were evaluated for antibacterial activity against *H. pylori* [Table 1]. The highest inhibitory activity was demonstrated by the EtOAc extract, which exhibited an inhibition zone with a diameter of 8.5 ± 3.1 mm (10 µg/mL), while hexane, dichloromethane, and butanol extracts did not exhibit any activity at this concentration. The EtOAc extract

**Table 1:** Anti-*H. pylori* activity of solvent extracts and fractions of acacia honey

Sample	Zone of inhibition diameter (mm) <sup>1)</sup>	
	Sample concentration (µg/mL)	
	10	20
Crude acacia honey (% v/v)	- 2	9.2 ± 5.8
<i>n</i> -Hexane extracts	--	--
Dichloromethane extracts	--	9.1 ± 5.5
Ethyl acetate extracts	8.5 ± 3.1 <sup>a</sup>	12.6 ± 8.0 <sup>a</sup>
<i>n</i> -Butanol extracts	--	--
Fr. A1	--	12.9 ± 7.8
Fr. A2	9.1 ± 0.6	13.2 ± 6.7
Fr. A3	10.5 ± 3.6	13.7 ± 9.1
Fr. A4	12.5 ± 6.3	15.1 ± 6.5
Fr. A5	16.0 ± 5.7 <sup>a</sup>	20 <sup>a</sup>
Fr. A6	10.5 ± 4.9	16.0 ± 6.5
Fr. A7	--	8.8 ± 2.8
Fr. A8	--	--
Fr. 51	11.3 ± 4.8	14.4 ± 7.1
Fr. 52	12.3 ± 6.5	15.1 ± 6.7
Fr. 53	13.5 ± 2.3	15.5 ± 6.5
Fr. 54	20 <	20 <

<sup>1</sup>Mean zone diameter after triplicate assay, mean ± standard deviation (SD).

<sup>2</sup>Below 8.0 mm for zone diameter. <sup>a</sup>Significant at the 0.05 probability level. Values are expressed as mean ± SE.

yielded eight fractions, and then four subfractions were subsequently obtained chromatographically. Among these fractions, Fr. 54 showed the most potent antibacterial effect against *H. pylori* [Table 1]. Compound I was isolated from Fr. 54 and its structure was identified based on spectroscopical data, chemical evidence, and comparisons with previous reports [Figure 1]. Its structure was elucidated as abscisic acid. Based on the absorption maxima in the UV spectra with three-dimensional UPLC-DAD detection, the monitoring wavelength was detected at 260 nm [Figure 2A]. The active compound I was identified by comparing the UPLC retention times and UV absorption of its target peaks in the EtOAc extract of the acacia honey with the standard compound. The UV spectrum is shown in Figure 2B.

### Determination of MIC and MBC of abscisic acid

The mean MICs of abscisic acid and amoxicillin (positive control) were 2.7 ± 1.3 and 10.9 ± 5.5 µg/mL, respectively [Table 2]. Therefore, the abscisic acid isolated from Korean acacia honey was approximately fourfold more effective than amoxicillin in inhibiting *H. pylori* in the *in vitro* tests. In addition, the mean MBC for abscisic acid (6.9 ± 1.9 µg/mL) was lower than that of amoxicillin (39 ± 7.5 µg/mL), suggesting that abscisic acid showed a more effective inhibition than amoxicillin did [Table 2].

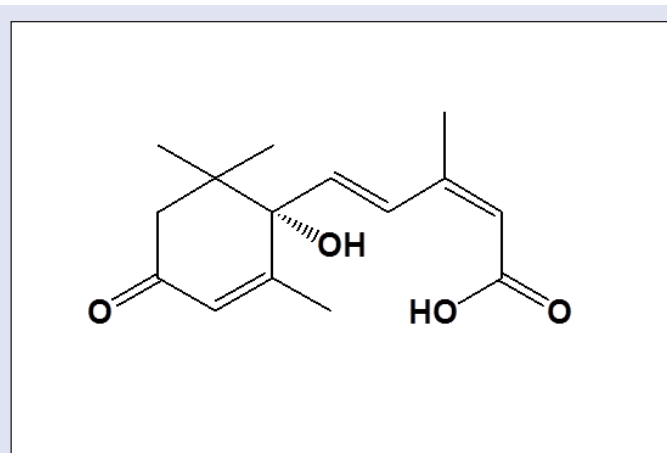
### Time-kill studies

The time-kill curves of abscisic acid against *H. pylori* are illustrated in Figure 3. The abscisic acid showed a concentration-dependent CFU reduction. All the concentrations tested exhibited antibacterial activity against *H. pylori* over time, and most concentrations reduced the bacterial count by least 8 log units after 24 h. At a concentration of 10.8 µg/mL (corresponding to 4 × MIC), 100% of the bacterial cells were killed within 36-72 h. However, there was bacterial cell growth at concentrations that were equivalent to 1× and 2 × MIC within 36-72 and 48-72 h, respectively.

**Table 2:** MIC and MBC of abscisic acid against *H. pylori*

	MIC (µg/mL)	MBC (µg/mL)
Abscisic acid	2.7 ± 1.3 <sup>b</sup>	6.9 ± 1.9 <sup>b</sup>
Amoxicillin	10.9 ± 5.5 <sup>a</sup>	39 ± 7.5 <sup>a</sup>

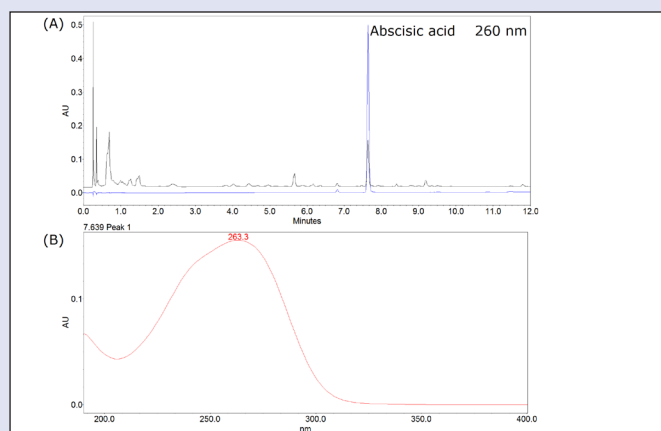
<sup>a</sup>Significant at the 0.05 probability level. <sup>b</sup>Significant at the 0.0001 probability level. Values are expressed as mean ± SE.

**Figure 1:** Chemical structure of abscisic acid isolated from Korean acacia honey

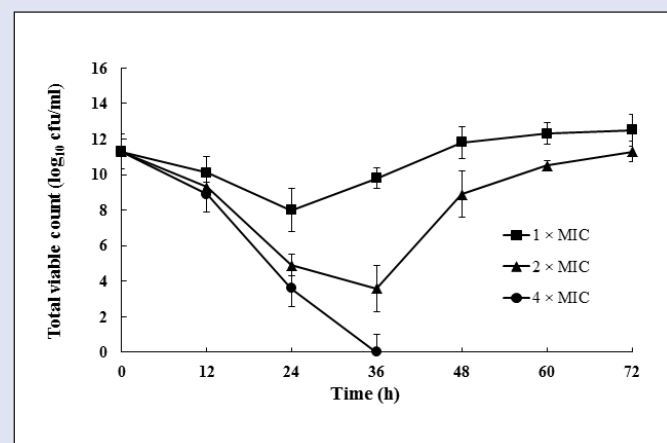
## DISCUSSION

In the preliminary screening, Korean acacia honey showed antibacterial activity against *H. pylori* at concentration as low as 10% (v/v). There are numerous reports on the antibacterial activity of natural honey, which is attributed to its acidity, osmolarity, hydrogen peroxide content, and phytochemical components (Mannina, 2015).<sup>[19]</sup> Honey is known to contain phytochemicals including peroxides, amylase, fatty acids, phenols, ascorbic acid, flavonoids, terpenes, and benzoic acid.<sup>[20]</sup> Honey is of plant origin and can be extracted with organic solvents. The active component content is often low or diluted in honey but concentrated following extraction, and the resulting extracts show higher activities.<sup>[21]</sup> These components could act as natural antibacterial agents or antioxidants and have been reported to inhibit the growth of *Streptococcus*, *Bacteroides*, *Staphylococcus*, *Prevotella*, and various other enteropathogens. Furthermore, the observation that honey produced in New Zealand and Saudi Arabia inhibit the growth of *H. pylori*<sup>[22,23]</sup> prompted our investigation of other honey varieties for potential antimicrobial effect. While the mechanism by which honey induces microbial death is considered to involve multiple pathways, its hydrogen peroxide content appears to be an important component mediating its antimicrobial effect. In addition, other research studies have found that the non-peroxide components of honey are extractable by organic solvents.<sup>[24]</sup>

In this study, organic solvents of varying polarities namely *n*-hexane, EtOAc, dichloromethane, and *n*-butanol were used to extract the anti-*H. pylori*-active principles in honey. Among the solvent fractions, those obtained from the EtOAc extract showed the highest activity against *H. pylori*, and abscisic acid was isolated from the most soluble EtOAc fraction. In this study, abscisic acid was isolated and identified in Korean acacia honey for the first time. Abscisic acid has been found in numerous varieties of honey including the Australian blue gum, Leatherwood, and acacia honeys from France, Germany, and Italy, and was revealed to have the potent anti-*H. pylori* effect. The positive control, amoxicillin, used in this study showed an anti-*H. pylori* effect with an MIC of 10.9 ± 5.5 µg/mL and MBC of 39 ± 7.5 µg/mL, whereas abscisic acid exhibited



**Figure 2:** (A) Typical chromatogram of EtOAc extract of Korean acacia honey and (B) UV spectrum of abscisic acid



**Figure 3:** Time-kill curve showing *in vitro* bactericidal effect of abscisic acid on *H. pylori*

MIC and MBC values of  $2.7 \pm 1.3$  and  $6.9 \pm 1.9$   $\mu\text{g/mL}$ , respectively. The bactericidal activity of the abscisic acid was determined using viability studies and was the highest at 4 $\times$  MIC concentration at 30–72 h. However, there was a recurrent growth of bacterial cells from 48 to 72 h after the cells were killed at 36 h at the lowest concentration (2 $\times$  MIC). This study demonstrated that the antibacterial activity of abscisic acid isolated from Korean acacia honey against *H. pylori* is evident at concentrations that can be achieved in the stomach following a reasonable oral dose. Furthermore, numerous people have ingested acacia honey, a common and popular floral-based food in Korea, in large quantities for a long time without any obvious adverse effects. Therefore, it is relatively safe. However, clinical trials are necessary to determine the efficacy and complete safety profile of Korean acacia honey for the treatment of dyspepsia, which is known to involve *H. pylori*.

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Nil

## Conflict of interest

There are no conflicts of interest

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