

Antibacterial Effects of Grape Extracts on *Helicobacter pylori*[∇]

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Anti-*Helicobacter pylori* activities were determined by agar dilution, confocal laser scanning microscopy, and cell proliferation assays following treatment with various grape extracts. Muscadine grape skin possessed the strongest activity, followed by grape synergy (skin and seed) and seed, suggesting that higher phenolic levels do not necessarily determine overall anti-*H. pylori* efficacy.

Helicobacter pylori is considered the etiological agent of peptic ulcers and gastritis and is associated with mucosa-associated lymphoid tissue lymphoma and gastric cancer (4). Although treatment is usually effective, it can result in side effects, such as antibiotic resistance development (14) and relapse due to low compliance (2). Therefore, alternative methods should be explored to treat *H. pylori* infection.

Studies have reported many natural plant extracts with anti-*H. pylori* activity, including garlic, broccoli, cranberries, and green tea (5, 12, 16, 26). Grapes (*Vitis vinifera*), well known for their high levels of antioxidants and polyphenols, have also shown promise as novel antimicrobial agents. A few studies have already reported the anti-*H. pylori* activities of grape seed and wine, including an active chemical constituent (e.g., resveratrol, a stilbene from red wine) (13). However, no effort has been made to evaluate the grape skin or different grape types (e.g., table and muscadine grapes). For example, muscadines (*Vitis rotundifolia*) contain significantly higher levels of phenolics than commercial table grapes in addition to possessing some unique forms of these compounds (23). However, little is currently known about the antibacterial properties these fruits possess, making them prime candidates for study. In addition, it is believed that the high complexity of bioactive compounds present in these products and their broad range of activity over a number of microorganisms may make it difficult for microbes to acquire resistance during treatment (26).

The objectives of this study were to investigate the effects of various grape extracts against *H. pylori* and to determine any correlations between anti-*H. pylori* activity and extract phenolic content.

Five *H. pylori* strains (G2-1, 26695, WV 99, NB2-1, and 1324P-1) were obtained from Douglas Berg (Washington University, St. Louis, MO), and five clinical *H. pylori* isolates (D5251, D5131, D5178, D5136, and D5135) were obtained from Ben Gold (Emory University and Centers for Disease Control and Prevention, Atlanta, GA). *H. pylori* P1pDH80, a green fluorescent protein (GFP)-labeled strain, was provided by Rainer Haas (Max von Pettenkofer Institut für Hygiene und Medizinische Mikrobiologie, München, Germany). *H. pylori*

SS1, a mouse-adapted strain, was provided by Kathryn Eaton (Department of Veterinary Biosciences, Ohio State University, Columbus, OH). For the entire study, bacteria were grown on brain heart infusion agar (Difco Laboratories, Detroit, MI) (pH 7.4 ± 0.2) supplemented with 10% horse serum (HS) (Sigma Chemical Co., St. Louis, MO) at 37°C for 72 h under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD) (8). Liquid cultures were grown in brucella broth (Difco) (pH 7.0 ± 0.2) supplemented with 10% HS and incubated, with shaking (60 rpm), under the same conditions. Cultures were transferred from frozen stock (brucella broth-HS-10% glycerol, -80°C), subcultured twice, and then grown overnight for antimicrobial susceptibility testing.

Fresh grapes were purchased from a local supermarket. Skins from red, white, and black table grapes were dried at 70°C for 3 days in a gravity convection oven (Fisher Scientific, Gaithersburg, MD). Muscadine powder was provided by Muscadine Products Corporation (Wray, GA). Samples were defatted in petroleum ether (VWR International, West Chester, PA) and extracted in acetone-water-acetic acid (90:9.5:0.5) at 60°C for 8 h using a Soxhlet apparatus (7). The crude extracts were concentrated under vacuum, resuspended in methanol, and filter sterilized (0.2 μm; VWR). All extracts were stored at -20°C in the dark and used within 1 week. The total phenolic content of each extract was determined by the Folin-Singleton colorimetric method (21) and expressed as mg gallic acid equivalents (GAE)/g extract dry weight (dw). Major phenolics were measured by high-performance liquid chromatography as previously described (20) and reported as mg/g extract dw.

The anti-*H. pylori* activities of grape extracts (range, 256 to 2,048 μg/ml) and the pure compounds resveratrol, ellagic acid, and myricetin (range, 6.25 to 50 μg/ml) (Sigma) were evaluated by the agar dilution method in duplicate (14a). Each bacterial suspension was spot inoculated (ca. 1 × 10⁵ CFU/spot) onto brain heart infusion-HS plates containing each antimicrobial, and the MIC was determined following incubation at 37°C for 3 days under microaerophilic conditions.

AGS cells (ATCC CRL-1739; American Type Culture Collection, Manassas, VA) were grown in Ham's F-12K medium supplemented with 10% fetal bovine serum (HyClone Laboratories, South Logan, UT) and incubated at 37°C in a 5% CO₂ incubator with 100% humidity to 90% confluence. Cells were washed with phosphate-buffered saline (PBS; pH 7.4) and de-

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TABLE 1. MICs of grape extracts and compounds on *H. pylori* growth by agar dilution assay

Extract/ compound ^a	MIC (µg/ml) of <i>H. pylori</i> strain ^b :											
	G2-1	26695	WV99	NB2-1	1324P-1	D5251	D5131	D5178	D5136	D5135	SS1	P1pDH80
RGS	1,024	512	512	512	512	1,024	512	512	512	512	—	—
WGS	1,024	512	1,024	512	512	1,024	512	512	512	512	—	—
BGS	1,024	512	1,024	1,024	512	2,048	512	512	512	512	—	—
MSN	256	256	256	512	256	256	512	512	256	512	512	512
MSD	512	1,024	512	256	256	256	1,024	1,024	512	1,024	512	1,024
MSY	512	512	1,024	512	1,024	512	512	1,024	512	1,024	512	512
RSV	—	12.5	—	—	—	—	—	—	—	—	6.25	12.5
ELA	—	50	—	—	—	—	—	—	—	—	12.5	12.5
MYR	—	>50	—	—	—	—	—	—	—	—	>50	>50

^a RGS, red grape skin; WGS, white grape skin; BGS, black grape skin; MSN, muscadine grape skin; MSD, muscadine grape seed; MSY, muscadine grape synergy; RSV, resveratrol; ELA, ellagic acid; MYR, myricetin.

^b Average MIC at 72 h by agar dilution assay. Each experiment was performed in duplicate. —, not determined.

tached using 0.25% trypsin-EDTA (Sigma). Cell concentrations were determined using trypan blue and a hemocytometer.

For cell viability assays, overnight cultures of *H. pylori* strains 26695, P1pDH80, and SS1 at final concentrations of 5×10^5 CFU/ml were treated with grape extract or pure compounds for different times (see Table 2). Following treatment, *H. pylori* was pelleted by centrifugation ($3,500 \times g$ for 10 min), washed twice with sterile PBS, and resuspended in fresh cell culture medium. AGS cells were grown as described previously, distributed into 96 cell culture wells (Corning Costar Corp., Cambridge, MA), and incubated overnight to allow for adherence. Extract-treated *H. pylori* cultures (26695, P1pDH80, and SS1) were distributed to each well at a bacterium-to-AGS-cell ratio of 100:1 (based on the control, i.e., AGS cells infected with *H. pylori* without treatment). This was necessary since AGS cell growth was sensitive to both extracts and constituent compounds in preliminary experiments (data not shown). Following overnight incubation, the culture medium was replaced with RPMI 1640 (HyClone Laboratories) after washing with sterile PBS to remove nonadherent *H. pylori* cells. AGS cell proliferation was then determined by adding 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (CellTiter 96 AQueous one-solution proliferation assay; Promega, Madison, WI) following the manufacturer’s directions. Absorbances were read at 490 nm using a BioTek µ-Quant microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) and standardized against the AGS cell control.

The effects of muscadine grape extracts on *H. pylori* attachment to AGS cells were also determined by confocal laser scanning microscopy. Overnight cultures of GFP-labeled *H. pylori* P1pDH80 were treated with different concentrations of muscadine grape extracts for 1, 2, 4, 6, 12, and 24 h under microaerophilic conditions. Following treatment, *H. pylori* cells were washed in sterile PBS and incubated for 1 h with overnight cultures of AGS cells attached to eight-well Labtek chamber slides (Fisher Scientific, Norcross, GA) at low density. Following the removal of nonadherent cells, the attachment of GFP-labeled *H. pylori* to host cells was examined using a Zeiss LSM-510 laser scanning confocal microscope (Carl Zeiss, Inc., Germany).

During storage, total phenolic levels in grape extracts declined significantly, with up to 46% of the phenolic content lost

during 3-month storage at 4°C and –15°C (data not shown). However, phenolic levels for grape extracts stored at –80°C (with or without N₂) did not significantly decrease ($P > 0.05$) within the first month. Due to the rapid loss of total phenolic compounds in grape extracts, all the experiments were carefully implemented as to maximize the retention of these bioactive compounds.

The MIC results indicate that the grape extracts and pure compounds have significant but differing effects against *H. pylori* growth (Table 1). Muscadine grape skin extract was most effective (MIC range, 256 to 512 µg/ml), followed by muscadine seed (MIC range, 256 to 1,024 µg/ml) and synergy extracts (skin and seed) (MIC range, 512 to 1,024 µg/ml). Resveratrol and ellagic acid also inhibited *H. pylori* (MIC range, 6.25 to 50 µg/ml); however, myricetin had no effect and was not tested further (Table 1).

Figure 1 shows that there were substantial reductions in AGS cell viabilities following 24-h *H. pylori* infection with all three tested strains as determined by MTS assay. However, following treatment of the cells with grape extracts, average AGS cell growth was significantly higher than the controls (Table 2) and directly proportional to both extract concentration ($P < 0.05$) and treatment time ($P < 0.05$). Muscadine grape skin extract had the highest anti-*H. pylori* activity following 24 h treatment (AGS cell growth range, 97.8 to 99.4% at 4,096 µg/ml), followed by muscadine grape synergy and seed extract (Table 2). Treatment of *H. pylori* 26695 and SS1 with

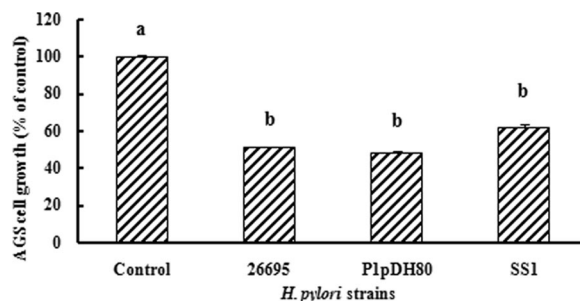


FIG. 1. AGS cell proliferation following 24-h exposure to *H. pylori*. Error bars represent the standard errors of eight replicates. The same letter represents no significant difference ($P > 0.05$) between *H. pylori* strains.

TABLE 2. AGS cell proliferation following infection with *H. pylori* 26695, SS1, and GFP-labeled P1pDH80 treated with muscadine grape skin, seed, and synergy extracts

Extract	Concentration (µg/ml)	% AGS cell proliferation at treatment time (h) ^a :														
		0			2			6			12			24		
		26695	P1	SS1	26695	P1	SS1	26695	P1	SS1	26695	P1	SS1	26695	P1	SS1
Skin	0	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 b	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a
	512	44.6 a	48.3 a	54.5 a	45.4 a	50.5 b	50.8 a	47.0 a	62.3 b	73.9 b	73.2 b	86.8 b	85.3 b	89.4 b	89.8 b	90.8 b
	1,024	44.6 a	48.3 a	54.5 a	45.3 a	47.7 a	55.8 b	55.4 b	72.8 c	84.2 c	84.7 c	92.4 c	90.7 c	94.4 b	94.2 c	95.4 c
	2,048	44.6 a	48.3 a	54.5 a	46.7 b	49.4 a	55.0 b	71.6 c	77.5 d	86.2 c	90.6 d	93.1 c	91.8 c	98.4 c	97.3 d	97.3 d
	4,096	44.6 a	48.3 a	54.5 a	48.3 b	52.4 b	55.9 b	73.5 c	78.3 d	90.8 d	95.9 e	94.2 d	95.2 d	99.4 c	97.8 d	98.8 d
Seed	0	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a
	512	44.6 a	48.3 a	54.5 a	41.8 a	48.2 a	59.0 b	45.0 a	52.4 b	63.0 b	60.0 b	57.6 b	80.3 b	65.7 b	62.5 b	85.0 b
	1,024	44.6 a	48.3 a	54.5 a	43.2 a	48.6 a	58.2 b	47.3 a	73.9 c	61.3 b	73.9 c	89.9 c	82.1 c	85.8 c	93.2 c	86.4 b
	2,048	44.6 a	48.3 a	54.5 a	46.7 b	54.2 b	58.9 b	47.8 b	75.0 c	74.8 c	73.1 c	92.4 c	84.5 c	90.7 d	94.5 c	92.5 c
	4,096	44.6 a	48.3 a	54.5 a	47.5 b	55.4 b	59.0 b	56.4 c	75.1 c	81.3 d	80.4 d	93.1 c	89.5 d	92.6 d	95.3 c	95.0 c
Synergy	0	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a	44.6 a	48.3 a	54.5 a
	512	44.6 a	48.3 a	54.5 a	43.5 a	48.4 a	55.0 a	50.1 b	58.3 b	55.8 a	76.1 b	87.9 b	84.2 b	84.5 b	92.2 b	85.5 b
	1,024	44.6 a	48.3 a	54.5 a	43.5 a	48.5 a	55.5 a	53.7 b	67.5 c	61.8 b	80.9 c	92.2 c	88.4 c	88.2 c	97.2 c	93.1 c
	2,048	44.6 a	48.3 a	54.5 a	43.9 a	48.4 a	56.8 a	60.8 c	76.4 d	74.0 c	88.2 d	94.7 c	88.6 c	92.9 c	98.2 c	95.1 c
	4,096	44.6 a	48.3 a	54.5 a	40.8 a	51.9 b	51.7 b	68.8 d	77.2 d	80.9 d	89.2 d	93.5 c	90.1 d	95.9 c	98.8 c	96.8 c

^a The same letter represents no significant difference ($P > 0.05$) between treatment concentrations for each *H. pylori* strain within each column for each extract. Eight replicates per extract concentration and time combination were used for each *H. pylori* strain.

resveratrol (6.25 to 50 µg/ml) and ellagic acid (6.25 to 50 µg/ml) was also effective (data not shown); however, no significant difference ($P > 0.05$) was found between AGS cell growth following treatment of these two *H. pylori* strains with these compounds.

Because bacteria were treated in the absence of AGS cells, *H. pylori* populations used for MTS assays varied. Therefore, AGS cells were exposed to different concentrations of *H. pylori* 26695 without muscadine grape extract treatment for 24 h and measured by MTS assay. AGS cell growth with extract-treated *H. pylori* (12 h) was then compared to a standard curve. Figure 2 shows that AGS cell growth was higher for extract-treated *H. pylori* 26695 at the same cell populations as controls, indicating that additional factors, besides reduced bacterial numbers, may affect *H. pylori* following treatment.

Treatment with muscadine grape skin extract resulted in a significant decrease in *H. pylori* attachment to AGS cells compared to that of controls (Fig. 3). All extracts (range, 1,024 to 4,096 µg/ml) reduced *H. pylori* attachment with effects observed at the lowest concentration (data not shown). The majority of the GFP-labeled *H. pylori* cells were coccoidal in form

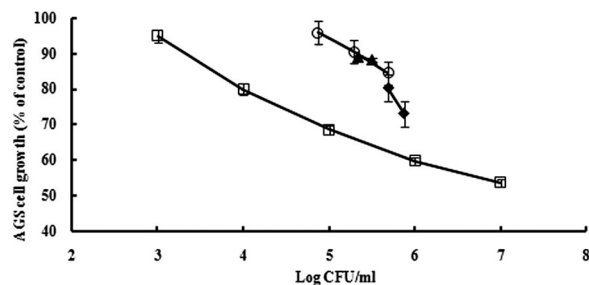


FIG. 2. AGS cell proliferation following 24-h exposure to various concentrations of *H. pylori* 26695 (◇) or *H. pylori* 26695 treated with muscadine skin (○), seed (◆), or synergy (▲) extracts for 12 h (one representative experiment with three replicates). Error bars represent standard errors.

and appeared to clump at higher extract concentrations and longer treatment times, especially at 12 and 24 h, indicating a decline in attachment efficiency (Fig. 3).

Phenolic compounds in grape products play important roles for antioxidant, anti-inflammatory, and antimicrobial effects (13, 18, 20). In this study, muscadine grape seed extract contained the highest total phenolic content (645.5 mg GAE/g dw), followed by muscadine synergy and skin extracts (381.9 and 135.0 mg GAE/g dw, respectively). Total phenolic concentrations in muscadine seed extracts were approximately five times higher than its skin counterpart, a finding consistent with that previously reported by Pastrana-Bonilla et al. (18). High-performance liquid chromatography results were also in agreement with the previous study for the major phenolics ellagic acid, myricetin, quercetin, and resveratrol with respective average concentrations of 77.3, 22.5, 3.4, and 1.4 mg/g extract dw.

In this study, muscadine grape skin extract was most effective against *H. pylori* in all tests performed. Although muscadine grape seed contained significantly higher total phenolic levels than other extracts, its efficacy against *H. pylori* was lower than or similar to that of muscadine skin and synergy extracts (Tables 1 and 2), suggesting that higher phenolic levels do not necessarily correlate with increased anti-*H. pylori* activity but rather the type and concentrations of compounds present in these extracts. Resveratrol, the least-abundant major phenolic in muscadine grape skin, has been shown to inhibit *cagA*-positive *H. pylori* strains (6). Ellagic acid, the most-abundant phenolic, has also been reported to possess anti-*H. pylori* activity (15). In addition, this compound has been found to inhibit cancer, alter bacterial protein conformations, and affect ion pump function (11, 22); however, it is not known if a similar mode of action against *H. pylori* exists. Because these phenolics are partially hydrophobic, this may allow them to interact with the bacterial cell wall and lipopolysaccharide interfaces more effectively by decreasing membrane stability (25). Therefore, further research on individual compounds and their interactions in the fruit itself is necessary to elucidate the possible

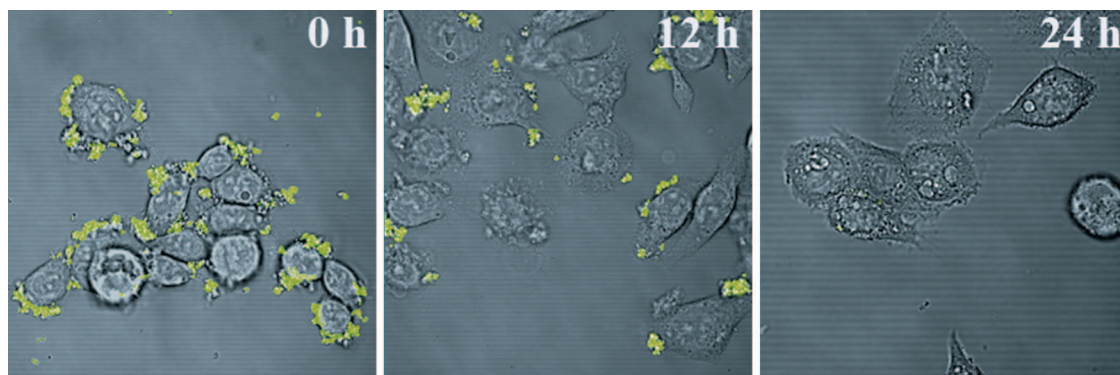


FIG. 3. Confocal laser scanning microscopy images of GFP-labeled *H. pylori* attached to AGS cells following treatment for 0, 12, and 24 h with muscadine grape skin extract at 4,096 $\mu\text{g/ml}$. Excitation wavelength, 488 nm; emission wavelength, 510 to 530 nm. All images were acquired and analyzed by the LSM-510 software package (Zeiss LSM 510, version 2.8).

synergistic/antagonistic mechanism(s) by which these compounds exert their effects.

Bacterial attachment to stomach epithelium is considered the initial step for *H. pylori* pathogenesis. Several recent studies have evaluated the antiadhesive properties of various natural compounds (1, 9, 17). Confocal microscopy revealed fewer bacterial cells present on host cell surfaces following treatment with grape extracts (Fig. 3), in agreement with AGS cell proliferation assays. Two possible explanations are that these compounds may interfere with *H. pylori*'s ability to increase the expression of AGS cell adhesion molecules (10) or inhibit sialic acid-specific adhesions to epithelial cells (1).

Following treatment with muscadine grape extracts or compounds, AGS cell proliferation was similar to that of the controls without *H. pylori* (Table 2), suggesting that extracts either reduce or inhibit *H. pylori* from binding to and/or damaging AGS cells. Figure 2 reveals that approximately 2 log CFU *H. pylori*/ml in extract-treated populations lost the ability to damage host cells, suggesting that some unknown factor(s), in addition to lower *H. pylori* numbers, may be responsible for higher cell proliferation levels. The loss of attachment potential for *H. pylori* following grape extract treatment could be due to many levels of damage and injury to the microorganism, including the inhibition of basic energy production and/or virulence factors. Because *H. pylori* VacA toxin is known to cause gastric injury via damage to host stomach cells, the increased AGS cell proliferation may be attributed to the inhibition of vacA-induced ion channel formation, urea conduction, and cell vacuolization (24). The polyphenol compounds may form aggregates with the toxin, in turn preventing its receptor binding and internalization into the host cell (27). A recent finding by Ruggiero et al. (19) found that red wine was able to prevent *H. pylori*-induced gastric epithelium damage in mice, suggesting that VacA inhibition may be involved. However, further studies are needed to determine the specific effect(s) (e.g., loss of attachment mechanisms, virulence factors, host membrane integrity, etc.) these extracts have on the organism's overall pathogenesis.

In this study, grape extracts and their compounds were effective at inhibiting *H. pylori* in vitro, with highest efficacy by muscadine grape skin extract. Although anti-*H. pylori* activities by individual compounds were reported, it is believed that a

synergistic mode of action is more likely responsible for the extract's antimicrobial activity (26). However, these complex interactions among compounds found in these extracts have yet to be determined. Our results suggest that anti-*H. pylori* activity does not necessarily correlate with higher phenolic content as previously thought. This study supports the need for further research to fully evaluate the in vivo potential of grape extracts and constituent compounds for use in the dietary management of *H. pylori* infection.

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