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Antibacterial Activity of Extracts of Halgho and Rashe Grape Cultivars against *Helicobacter pylori*

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One of the main causes of failure in treatment of *Helicobacter pylori* (*H. pylori*) infection is its antibiotic resistance. Grape cultivars contain different useful components including phenolic compounds with antimicrobial effects. In the present study, the effects of two grape varieties; Halgho red grape and Marivan black grape (Rashe), have been evaluated against the clinical isolates of *H. pylori*. The effect of the seed, skin, and seed + skin extracts of the two varieties against *H. pylori* was evaluated using the disc diffusion and agar dilution methods. The stability of the antibacterial effects of the extracts under different temperature and pH values, and in the presence of Trypsin enzyme was investigated. The highest inhibitory zone diameter was observed for the seed extracts of Halgho and Rashe as 15 and 14.5 mm, respectively. The lowest Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were observed for the seed extracts of Halgho and Rashe (2048 μg/ml). Moreover, the antibacterial effects of the extracts of both varieties were preserved under the above-mentioned physicochemical conditions. Grape seed and skin extracts of the varieties studied have antibacterial effects against *H. pylori*. The effect was preserved under different physicochemical conditions.

Key words: *Helicobacter pylori*; Halgho grape; Rashe grape; Antibacterial activity.

Helicobacter pylori is a Gram-negative, microaerophilic spiral bacterium, living in the stomach. It is known as the cause of gastritis and peptic ulcer, and is associated with mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer[1]. Contamination of more than half of the world population by the bacterium makes it one of the most prevalent bacterial infections among human [2]. In spite of availability of appropriate antibiotic treatments, the potential of antibiotic resistance has increased for drugs such as metronidazole, tetracycline, amoxicillin, and clarithromycin that are commonly prescribed for the disease [3, 4].

Some previous studies have shown that taking fruits and vegetables rich in specific vitamins, antioxidants, and bioactive compounds such as phytochemicals would lead to a statistically significant reduction in the incidence of *H. pylori* infection or improvement in associated symptoms [5-9].

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Grape contains unique bioactive compounds, particularly major phenolic compounds such as ellagic acid, myricetin, quercetin, trans-resveratrol, and gallic acid [10]. The studies have shown that grape (*Vitis vinifera*) and the bioactive components present in its extract, particularly the polyphenols, have anti- *H. pylori* activity [11, 12]. Furthermore, in the studies on mouse model, the extracts reduce the inflammation and gastritis caused by *H. pylori* [13-16]. Previous studies have shown that among the grape varieties grow in Iran, red and black grape varieties have the highest content of trans-resveratrol [17]. This compound has wide ranging antimicrobial activity [18, 19].

Halgho red grape (*Vitis vinifera* Halgho) and Marivan black grape (*Vitis vinifera* Rashe) are native to Kurdistan region, Iran. So far, the antimicrobial activity of these varieties against *H. pylori* has not been evaluated. Therefore, in the present study, the antibacterial activity of seed, skin, and seed + skin extracts of these two grape varieties against *H. pylori* were evaluated. Furthermore, stability of the antimicrobial effect under different physicochemical conditions was studied.

MATERIALS AND METHODS

Grape collection and preparation of the extracts

Two varieties of Halgho red grape and Marivan black grape (Rashe) were obtained from a local market and the varieties were confirmed in the Agricultural Research Center, Kurdistan Province, Iran. Then, to prepare the extract, the skin and seed of the grapes were manually separated. The seed and skin were dried at 30 ºC in dark, and then powdered. The seeds were defatted in petroleum ether and then immersed in acetone: water: acetic acid (90:9.5:0.5) for 24 hours, filtered through Whatman No.1 filter paper, and concentrated in a rotary evaporator (Heidolph, Germany). Then, the concentrate was spread in glass plates at the room temperature in dark, under vacuum condition. After 24 hours, the solvent was removed completely and the powder extract was obtained. The extract was diluted in ethanol and dimethyl sulfoxide (DMSO) (Merck, Germany) at the ratio of 1:1. The extract was sterilized by 0.2-µm filter (Orange, Belgium) and was kept under -20 ºC until the experiments [20, 21].

Preparation of *H. pylori* isolates

Three clinical isolates of *H. pylori* were obtained from Kurdistan University of Medical Sciences. The isolates were grown on the brucella agar (Merck, Germany) supplemented with 10% defibrinated sheep blood and 7% fetal bovine serum (FBS) (Sigma, USA) at 37 ºC for 72 hours under microaerophilic condition (10% CO_2 ,5% O2, 90% humid) in an CO_2 incubator (Memmert, Germany)[21].

Determination of inhibition zone diameter

Firstly, the seed, skin, and seed + skin extracts of the two grape varieties were prepared at the concentration of 10000 µg/ml. Then, 50 µl of each extract was added to sterile blank discs (6 mm in diameter) (Padtan Teb, Iran) and the discs were kept in an incubator (Binder, USA) at 25 ºC. The discs were completely dried and the solvent was removed after 24 hours. An inoculum of each *H. pylori* isolate was prepared in a brucella broth (Merck, Germany) by adjusting the turbidity to that of a 1.0 McFarland Standard (3×10^8 CFU/mL) and cultured on the Brucella agar plates (supplemented with 10% defibrinated sheep blood and 7% FBS) using a sterile swap. Then, the extract discs were placed on the medium. The disc containing the solvent (ethanol: DMSO) was used as the negative control, while standard antibiotic discs of tetracycline (30 µg/disc), amoxicillin (25 µg/disc), and gentamycin (10 µg/disc) (Padtan Teb, Iran) were used as positive controls. The plates were kept in CO_2 incubator for 72 hours. The mean diameter of inhibition zone was measured in mm by a caliper [22].

Determination of MIC and MBC values of the extracts

To determine the MIC and MBC using the agar dilution method, the concentrations of zero (as the negative control, which contained the solvent without the extract) and 64-8192 µg/ml were prepared from the seed, skin, and seed + skin extracts of the two grape varieties. Then, for each extract, 1 ml of the extract was added to 19 ml melted brucella agar containing blood and FBS. In the following, from the fresh culture of *H. pylori*, concentration of 3×10^8 was prepared in brucella broth and 100 µl of the suspension was added to the media containing different concentrations of the extracts, spread over the medium by a hooked Pasteur pipette, and cultured for 72 hours in a CO_2 incubator. The experiments were performed in

triplicate and the lowest extract concentration at which the bacterium showing no growth was considered as the MIC, while MBC was determined by no bacterial growth following inoculation from the MIC plates and before that to the brucella agar no containing the extract [20].

Stability of anti-*H.pylori* effect of the extracts under different physicochemical conditions

Temperature

Of the seed, skin, and seed + skin extracts of the two grape varieties, 1000 µg/ml were kept at 50 and 80 ºC for 30 min and 121 ºC for 20 min. Then, 50 µl of the solution of all the extracts was added to sterile blank discs measuring 6mm in diameter. When the discs were dried, disc diffusion method was performed [22].

Trypsin enzyme

Considering the presence of trypsin in the stomach, we evaluated the extract stability when exposed to the enzyme. To this end, trypsin powder (Sigma, USA) was dissolved in distilled water to achieve the concentration of 1 mg/ml. Then, 100 µl of the enzyme solution was added to the seed, skin, and seed + skin extracts of the two grape varieties. After three hours, 50 µl of the solution was added to sterile discs (6mm in diameter) and when the discs were dried, disc diffusion method was applied [22].

The pH value

Firstly, the pH value of phosphate buffer solution (PBS) reached the values of 5, 7, and 8 using HCl and NaOH, with a pH-meter (Crison, Spain). Then, 100 µl of the solution was added to 100 µl of the extracts (at the concentration of 10000 µg/ml). Three hours later, 50 µl of the solution was added to two sterile discs measuring 6mm in diameter. When the discs dried, the disc diffusion method was used [22].

Statistical analysis

The data was analyzed by t-test using SPSS software, version 18. *P* value less than or equal to 0.05 was considered statistically significant.

RESULTS

Mean inhibition zone diameter with and without application of physicochemical factors

The mean diameter of inhibition zone for extracts of the two grape varieties (Halgho and Rashe) with and without temperature, pH, and enzymatic treatments are shown in Fig. 1 and Table 1.

The highest inhibition zone was observed for seed extracts of the two grape varieties, followed by the seed + skin extracts of the two varieties. The lowest inhibition zone diameter was observed for the skin extracts of the two varieties. According

Table 1. Mean diameter of inhibition zone for discs of grape extracts and antibiotics and the results obtained for effect of physicochemical factors on antimicrobial activity of the extracts. Mean diameter of the inhibition zone of the discs (measuring 6mm in diameter) containing the extracts and the solvent, and standard antibiotic discs of amoxicillin (25 µg), tetracycline (25 µg), and gentamycin (10 µg) were reported for duplicate experiments

No.	Extract and antibiotic discs	Mean diameter of inhibition zone (mm)							
		Without treatment	50 ºC	80 ºC	121 ºC	pH= 5	pH= 7	pH= 8	Trypsin
1	Halgho seed	15	15	15.5	14	15	15	14.5	15
2	Halgho skin	11	11	11	10.5	11	11	10	11
3	Halgho seed + skin	13.5	13.5	13.5	13	13.5	13.5	14	13
4	Rashe seed	14.5	14	14.5	15	14.5	13.5	14.5	14
5	Rashe skin	8.5	8.5	9	9	8	8.5	8.5	8.5
6	Rashe seed + skin	12	12	12	12	12	11	12	11.5
7	Tetracycline	67	*	*	*	*	*	*	*
8	Amoxicillin	75	*	*	*	*	*	*	*
9	Gentamycin	47	*	*	*	*	*	*	*
10	Solvent disc	ND	*	*	*	*	*	*	*

ND: No diameter for inhibition zone
*: Not studied

to the statistical analysis results, the mean inhibition zone diameter for Rashe seed extract was significantly higher than that for the Rashe skin extract (P= 0.01). However, the mean inhibition zone diameters of Halgho seed and skin extracts were not significantly different (P= 0.1). Other extracts were not significantly different with regard to the inhibition zone diameter produced (P>0.05). Following application of temperature, pH, and enzymatic treatments, the anti-*H. pylori* activity of the extracts for the two grape varieties were preserved (P>0.05). The three bacterial isolates were susceptible to tetracycline, amoxicillin, and gentamycin, and diameters of the inhibition zone produced by the antibiotics were higher than those produced by the extracts (P<0.05).

MIC and MBC results

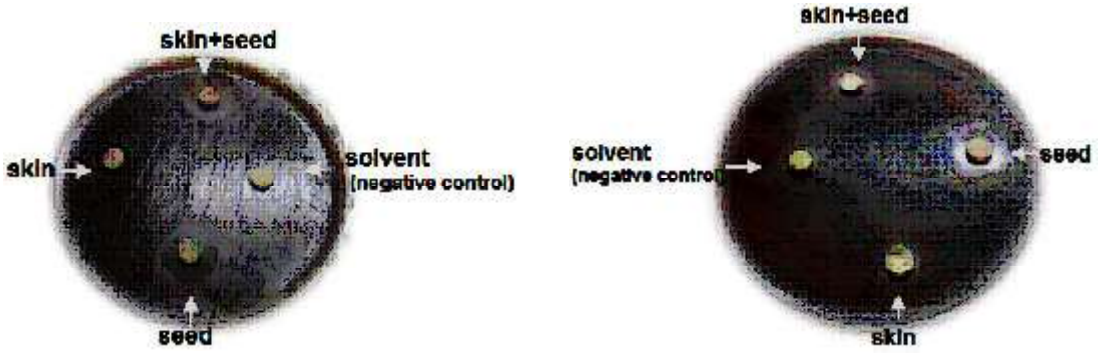
The MIC and MBC values obtained for extracts of the two grape varieties are provided in Table 2.

The highest antibacterial activity was observed for the seed extracts of the two varieties and seed + skin extracts of Halgho grape with the MIC value of 2048 µg/ml, followed by the Rashe seed + skin extract with the MIC value of 4098 µg/ml. The Rashe skin extract did not show any growth inhibitory effect against *H. pylori*. Moreover, in zero concentration of the extract, no growth reduction was observed. The MBC values obtained for the extracts were equal to the MIC values (Table 2).

Table 2. MIC and MBC values obtained for seed, skin, and seed + skin extracts of Rashe and Halgho grape varieties

Grape extract	MIC (µg/ml)	MBC (µg/ml)
Rashe seed	2048	2048
Rashe skin	*	*
Rashe seed + skin	4096	4096
Halgho seed	2048	2048
Halgho skin	8192	8192
Halgho seed + skin	2048	2048

*: MIC and MBC were not obtained for the concentrations studied.



a) Mean diameter of inhibition zone for Halgho grape extracts

b) Mean diameter of inhibition zone for Rashe grape extracts

Fig. 1. Mean diameter of inhibition zone for the grape extracts

DISCUSSION

Considering the increasing antibiotic resistance of *H. pylori* and the adverse effects of conventional treatments of the bacterium, making use of the plants with medicinal properties in treatment of the infection have been considered [23]. In previous studies, the anti-*H. pylori* properties of some plants, e.g., licorice and olive, have been demonstrated [24, 25]. Grape (*Vitis vinifera*) is native to south Europe and west Asia, and is currently cultivated in all parts of the world in various temperature ranges. The plant contains unique bioactive components such as catechin, epicatechin, and trans-resveratrol [26]. Some previous studies have confirmed the antibacterial effects of some of these components, especially trans-resveratrol, and extracts obtained from

different parts of the plant under *in vitro* and *in vivo* conditions [18, 27, 28].

Few studies have shown the anti-*H. pylori* effects of grape, its components, and products. Brown *et al*. (2009) reported the anti-*H. pylori* effects of skin, seed, and skin+ seed extracts of muscadine grape and the purified grape components such as resveratrol, ellagic acid, and myricetin with the MIC range of 6.25-1024 µg/ml [20]. Brown *et al*. (2010) demonstrated the anti-*H. pylori* effect of quercetin extracted from the grape with the MIC values of 64-128 µg/ml. Furthermore, they showed that muscadine skin extract and quercetin reduce the level of some inflammatory cytokines produced by *H. pylori* infection in mice [29].

In the study performed by Martini *et al*. (2009), the antibacterial effects of three grape varieties; Colorino, Sangiovese, and Cabernet Sauvignon, against *H. pylori* with the MIC values of 1.35-4.0 mg/ml were shown. In the present study, the seed extracts of Halgho and Rashe grapes and seed + skin extract of Halgho grape showed the highest anti-*H. pylori* effect with the MIC and MBC values of 2048 µg/ml. Moreover, it was observed that the anti-*H. pylori* effect of Rashe seed extract was significantly higher than that in the Rashe skin extract. The difference could probably be explained by the difference in chemical composition of various parts of the plant as well as the difference in components of various grape cultivars [30].

Zaidi *et al*. (2009) reported that resveratrol reduce the induction of IL-8 and reactive oxygen species (ROS) following exposure of *H. pylori* to gastric cancer cells [31].

In the current study, it was shown that the skin, seed, and seed + skin extracts of Halgho and Rashe grapes have anti-*H. pylori* activity. The highest anti-*H. pylori* activity was observed for the seed extracts. This is different from the results obtained by Brown *et al*. (2009) on evaluation of the antibacterial characteristics of muscadine grape against clinical isolates and standard strains of *H. pylori*. They reported the highest antibacterial activity for the grape skin extract [20]. The difference between the results could be explained by the difference in the grape varieties and bacterial isolates used in the studies. The grape extracts evaluated in the current study preserved their anti-*H. pylori* effect at 121 ºC for 20 min, pH value= 5, and in the presence of trypsin enzyme. Considering the acidic pH and presence of trypsin enzyme in the stomach, resistance to these physicochemical conditions has great importance. While in oral administration, the activity of some antibiotics is reduced in the acidic pH of the stomach, amoxicillin shows the highest activity in neutral pH values [32].

CONCLUSION

In general, for the first time it was shown that the seed and skin extracts of Rashe and Halgho grape varieties are effective against *H. pylori* under *in vitro* conditions. Therefore, identification of active components of these varieties and *in vivo* and *in vitro* evaluation of their anti-*H. pylori* effects would be helpful in prevention and reduction of the pathogenicity, or even treatment of the bacterium.

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