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Investigation of Bioactive Components, Antioxidant and Antimicrobial Activities of Traditional Turkish Beverage Hardaliye
Geleneksel Türk İçeceği Hardaliyenin Biyoaktif Bileşenleri ile Antioksidan ve Antimikrobiyal Aktivitelerinin İncelenmesi

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ABSTRACT

Objective: In our study, it was aimed to make the chemical analysis of hardaliye products (H1 and H2), which are commercially available by different manufacturers, and to examine their antioxidant and antimicrobial activities.
Methods: Antioxidant activity, organic acid and phenolic compounds, and antimicrobial activity in Hardaliye products (H1 and H2) were determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, Liquid Chromatography-High Resolution Mass Spectrometry (LC-HR/MS) and liquid microdilution methods, respectively.
Results: Ascorbic acid and fumaric acid from organic acids were determined by chemical analysis of hardaliye samples by LC-HR/MS method. While ascorbic acid concentrations were 21.295 mg/L and 26.84 mg/L in H1 and H2, respectively, fumaric acid concentrations were 59.55 mg/L in H1, and 224.562 mg/L in H2. While the phenolic component with the highest concentration in H1 was resveratrol (44.57 mg/L), it was observed that the phenolic component with the highest concentration in H2 was p-coumaric acid (31.87 mg/L). In terms of antioxidant activity, DPPH free radical scavenging activity of hardaliye samples was determined as 2.07±0,004% and 2.49±0,004% in H1 and H2, respectively. It was determined that hardaliye samples showed inhibitory effect (H1 MIC: 15.625 µg/ml, H2 MIC: <3.9 µg/ml) against only *S. epidermidis* ATCC 49461 strains among the tested microorganisms.
Conclusion: In our study, it was determined that two different commercial Hardaliye products contained very low concentrations of phenolic compounds compared to the data in the literature, and therefore it was thought that tested Hardaliye products did not show antioxidant activity.
Keywords: Hardaliye, phenolic component, antioxidant

ÖZ
Amaç: Çalışmamızda farklı üreticiler tarafından ticari olarak satışa sunulan geleneksel Türk içeceği hardaliye ürünlerinin (H1 ve H2) kimyasal analizinin yapılması, antioksidan ve antimikrobiyal aktivitelerinin incelenmesi amaçlanmıştır.
Yöntemler: Hardaliye ürünlerinde (H1 ve H2) antioksidan aktivite 1,1-diphenyl-2-picrylhydrazyl (DPPH) serbest radikal giderim aktivitesiyle, organik asit ve fenolik bileşenler Sıvı Kromatografi-Yüksek Çözünürlüklü Kütle Spektrometre (Liquid Chromatography-High Resolution Mass Spectrometry, LC-HR/MS) yöntemiyle, antimikrobiyal etkinlik sıvı mikrodilüsyon yöntemleriyle araştırılmıştır.
Bulgular: LC-HR/MS yöntemiyle hardaliye örneklerinin kimyasal analizi yapılarak organik asitlerden askorbik asit ve fumarik asit saptanmıştır. Askorbik asit konsantrasyonları H1 ve H2'de sırasıyla 21.295 mg/L ve 26.84 mg/L iken, H1'de fumarik asit konsantrasyonları 59.55 mg/L, H2'de ise 224.562 mg/L olarak saptanmıştır. H1'de konsantrasyonu en yüksek fenolik bileşen resveratrol (44.57 mg/L) iken, H2'de konsantrasyonu en yüksek fenolik bileşenin p-kumarik asit (31.87 mg/L) olduğu gözlenmiştir. Antioksidan etkinlik açısından hardaliye örneklerinin DPPH serbest radikal giderim aktivitesinin H1 ve H2'de sırasıyla %2.07±0,004 ve %2.49±0,004 olduğu tespit edilmiştir. Hardaliye örneklerinin test edilen mikroorganizmalardan sadece *S.epidermidis* ATCC 49461 kökenine karşı inhibitör etki (H1 MİK: 15.625 µg/ml, H2 MİK: <3.9 µg/ml) gösterdikleri saptanmıştır.
Sonuç: Çalışmamızda ticari olarak üretilen iki farklı hardaliyenin literatürdeki verilere kıyasla fenolik bileşenleri oldukça düşük konsantrasyonlarda içerdiği saptanmış olup, bu sebeple antioksidan aktivite göstermediği düşünülmüştür.
Anahtar kelimeler: Hardaliye, fenolik bileşen, antioksidan

Introduction

Plants produce the main metabolites necessary for their growth and development. Furthermore, it is known that plants produce bioactive components known as phytochemicals that have beneficial effects on human health as a result of their secondary metabolism. Phenolic compounds formed by the attachment of one or more hydroxyl groups to the benzene ring constitute a crucial part of phytochemicals (1).

Phenolic compounds are found in many parts of plants, such as stems, leaves, and flowers, in various fruits and vegetables, and beverages such as green tea (1). Grape, which is a fruit, is among the richest fruits in terms of phenolic components. These phenolic compounds are distributed in various parts of the grape. It is known that the phenolic components in grape juice are mainly obtained from grape skins and seeds, and to a lesser extent from the juicy parts of this fruit. The components and amounts of phenolic compounds are related to many factors (type of grape, the soil, geographical location, climatic conditions, harvest time, etc.) (2).

Phenolic compounds have important effects on human health as well as their functions such as taste and aroma in fruits and vegetables. It is known that phenolic compounds can bind free radicals and chelate with metals with their antioxidant effect. In addition, they can utilize anti-inflammatory, anti-allergic, anti-carcinogenic, anti-hypertensive and anti-microbial effects, and modulate the intestinal microbiota by acting as a prebiotic (1, 3). Due to these beneficial effects on human health, foods rich in phenolic components have become the focus of the field of nutrition.

Hardaliye is a non-alcoholic fermented beverage made from dark-colored grapes (Cabernet, Merlot, Shiraz, etc.). It is presumed that hardaliye production in Kirklareli and the region of Thrace has a history of nearly one and a half centuries. It can be made at home as well as commercially available. It is obtained by adding benzoic acid and crushed mustard seeds to crushed dark-colored and fragrant grapes and undergoing lactic acid fermentation. Benzoic acid prevents or reduces alcohol formation by acting on yeasts. Allyl isothiocyanates in the mustard seed structure largely create the distinctive aroma of mustard. It has also been shown to reduce alcohol formation by reducing yeast activity. Studies have reported that allyl isothiocyanate has antimicrobial activity as well as anti-cancer effect. In people who consume hardaliye, it has been reported that there is a significant decrease in diene conjugate, malondialdehyde and homocysteine concentrations due to the antioxidant capacity arising from the phenolic components contained in hardaliye. Interest in this drink has increased with results from clinical trials (4, 5).

In our study, we aimed to evaluate the chemical analysis, antioxidant and antimicrobial activities of the traditional Turkish beverage hardaliye products sold commercially by DPPH free radical scavenging activity, LC-HR/MS and broth microdilution, respectively.

Methods

Hardaliye Samples

Hardaliye – 1 (H1) and Hardaliye – 2 (H2), produced by different manufacturers and sold commercially, were purchased. Before use, test materials were passed through 0.45 µm filters and stored at + 4°C until use.

Analysis of Phenolic Compounds by LC-HR/MS

Phenolic compounds in Hardaliye samples were determined by LC-HR/MS method. LC-HR/MS experiments were performed by a Thermo Orbitrap Q-Exactive ESI Mass Spectrometry system (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The samples were separated on a C18 (150x3 mm; 3 µm) column (Fortis Technologies, UK) at 25°C. The chromatographic conditions, particularly the composition of the mobile phase and its pH, were optimized through several trials to achieve good sensitivity and symmetric peak shapes of analytes. For that purpose, at various flow rates different solvents of mixtures, such as methanol, acetonitrile, formic acid and acetic acid were tested. The best results were acquired

using methanol: formic acid as the mobile phase and was applied to the gradient program. The mobile phase was a mixture of mobile phase A (1% formic acid solution in water) and B (1% formic acid solution in methanol), the gradient program of which was 0-1.00 min 50% A and 50% B, 1.01-3.00 50% A and 50% B, 3.01-6.00 0% A and 100% B, 6.01-7.00 min 50% A and 50% B and finally 7.01-10.00 min 50% A and 50% B. The flow rate of the mobile phase was 0.35 mL/min. The injection volume was 10 µL. The dihydrocapsaicin was used as an internal standard.

Determination of Antioxidant Activity

In this study, the antioxidant effect of H1 and H2 was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Sigma Aldrich, Germany) (6). DPPH is a stable free radical with characteristic absorption at 517 nm. DPPH solution when freshly prepared is dark purple and gives maximum absorbance at 517 nm.

The presence of antioxidant activity was evaluated in proportion to the decrease of DPPH's absorbance value at 517 nm. H1 and H2 were dried using a lyophilizer. DPPH solution at a concentration of 40 µg/mL was added to the solutions prepared with ethanol at concentrations of 10, 25, 50, and 100 µg/mL. Ethanol was used as a control. After 30 min. of incubation at room temperature, in the dark, at 517 nm, absorbances were measured in a spectrophotometer (Synergy H1 Hybrid Reader, BioTek, U.S.A). The absorbance values of the samples were evaluated against the control. Free radical scavenging activity was calculated using the following equation.

DPPH Radical Scavenging Activity (% inhibition) = [(A control-A sample) / (A control)] × 100

(A control is the absorbance of the control; A sample is the absorbance of the sample).

Investigation of Antimicrobial Efficacy with Minimum Inhibitor Concentration (MIC)

Standard Strains Used in the Study

In our study, the antimicrobial activities of hardalive samples were determined against for Gram-positive with *Staphylococcus aureus* ATCC 25923, *S. epidermidis* ATCC 49461, *Bacillus cereus* ATCC 14579, *Enterococcus faecalis* ATCC 29212; for Gram-negative with *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 70063, *Acinetobacter baumannii* ATCC 19606, *Helicobacter pylori* ATCC 43504; for yeasts *Candida albicans* ATCC 66027 and *C. glabrata* ATCC 2001.

The standard strains were cultured in Sabouraud Dextrose Agar, 5% Sheep Blood Agar, Mac Conkey agar and Columbia agar [(10% defibrinated horse blood and supplement with Vancomycin (10 mg/L), Cefsulodin (5 mg/L), Trimethoprim (5 mg/L) and Amphotericin B (5 mg/L)] for *Candida* species, Gram-positive strains, Gram-negative strains and *Helicobacter pylori*, respectively.

Antibacterial Efficacy

Resazurin Microtiter Assay (REMA)

The resazurin microplate method was used to determine the antibacterial activities and MIC of H1 and H2 against standard bacterial strains and the study was repeated twice. Streptomycin (Sigma Aldrich, Germany) was used as the standard drug. Stock solutions of the studied samples at a concentration of 1000 µg/mL were prepared with DMSO and passed through membrane filters with a diameter of 0.22 µm. 50 µl of Brucella broth (BD BBL, USA) for *H. pylori* and 50 µl of Mueller Hinton Broth (Merck, Germany) for other bacteria were dispensed into the microplates. Serial dilutions of the prepared solutions were made by adding 1000 µg/mL to the first wells of the microplates and the MIC range was set as 3.9-1000 µg/mL. Serial dilutions were made by setting the final concentration of streptomycin as 83 µg/mL and adding 50 µl to the first well. Serial dilutions were made by placing dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) as a negative control in one column of microplate and 50 µl of standard bacteria as a positive control in one column. 3 McFarland in

Brucella broth containing 10% Fetal Bovine Serum (Lonza, USA) from colonies of *H. pylori* and 0.5 McFarland standard in Mueller Hinton Broth from other strains were prepared and diluted 1:100. 10 µl of the prepared suspensions were added to the wells. Plates were covered with parafilm, microplates belonging to *H. pylori* were incubated for 72 hours at 37°C in the microaerophilic environment (Thermo Scientific™ Oxoid™ CampyGen™, UK), and others at 37°C for 24 hours in aerobic environment. After incubation, 33.75 mg of resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide) (Sigma Aldrich, Germany) and 20% Tween 80 (Merck, Germany) dissolved in 5 mL distilled water were added to all wells. 10 µl was added, plates were left to incubate for 2-4 hours and the results were evaluated visually. The lowest concentration preventing the color change from purple to pink was determined as the MIC value.

Antifungal Efficacy

Resazurin Microtiter Assay (REMA)

The resazurin microplate method was used to determine the antifungal activities and MIC of H1 and H2 against standard yeast strains and the study was repeated twice. Fluconazole (Sigma Aldrich, Germany) was used as the standard drug. Stock solutions of the studied samples at a concentration of 1000 µg/mL were prepared with DMSO and passed through membrane filters with a diameter of 0.22 µm. 50 µl of Mueller Hinton Broth was distributed to each well, serial dilutions of the prepared solutions were made by adding 1000 µg/mL to the first well and the MIC range was set to 3.9-1000 µg/mL. Serial dilutions were made by setting the final concentration of fluconazole as 30 µg/mL and adding 50 µl to the first well. Serial dilutions were made by placing DMSO as a negative control in one column of the microplate and 50 µl of standard strains as a positive control in another column. Suspensions equivalent to 0.5 McFarland standard were prepared from fresh yeast colonies and diluted 1: 100. 10 µl of the prepared suspensions were added to the wells. Plates were covered with parafilm and incubated at 37°C for 48 hours in an aerobic environment. After incubation, 10 µl of 33.75 mg of resazurin dissolved in 5 mL of distilled water and 20% Tween 80 were added to all wells, the plates were left to incubate for 12-24 hours and the results were evaluated visually. The lowest concentration preventing the color change from purple to pink was determined as the MIC value.

Results

Contents of Phenolic Compounds in Hardaliye Products

In our study, the chemical analysis of commercially sold hardaliye products was made by LC-HR/MS method and 28 components were determined. The phenolic components and their amounts (mg/L) detected in the hardaliye samples are given in Table 1. In Figure 1, some LC-HR/MS chromatograms of H1 and H2 are shown.

In the study, ascorbic acid and fumaric acid were determined as organic acids in both hardaliye products. Ascorbic acid was detected as 21.295 mg/L in H1 and 26.84 mg/L in H2. It was observed that fumaric acid with the highest concentration was 59.55 mg/L in H1 and 224.562 mg/L in H2 (Table 1).

Among the phenolic components, the highest amount of resveratrol, (-)-epigallocatechin, hyperoside, (+)-catechin, (-)-epicatechin, quercetin and myricetin were detected in H1, with concentrations of 44.57, 7.108, 5.067, 4.809, 4.023, 3.305, and 1.689 mg/L respectively. The highest number of phenolic components in H2 were p-coumaric acid, resveratrol, (-)-epicatechin, (+)-catechin, (-)-epigallocatechin, caffeic acid and their concentrations were 31.87, 16.64, 9.428, 5.381, 2.5859, and 1.559 mg/L, respectively (Table 1).

Antioxidant Activity of Hardaliye Products

DPPH free radical scavenging activity was studied at four different concentrations (10, 25, 50, 100 µg/mL). Antioxidant effect comparisons were made with BHA (Butylated hydroxy anisole) (Sigma Aldrich, Germany) used as standard. At a concentration of 100 µg/mL, an

inhibition rate of 2.07±0,004% and 2.49±0,004% was observed in H1 and H2, respectively. Inhibition values of standard substances and samples are shown in Figure 2.

Antimicrobial Activity of Hardaliye Products

The antimicrobial activities of H1 and H2 on Gram-positive and Gram-negative bacteria and yeasts were performed by broth microdilution method, and the microorganisms and MIC results are given in Table 2. Antibacterial activity of H1 and H2 on *S. epidermidis* ATCC 49461 was observed as MIC: 15.625 µg/mL, MIC: <3.9 µg/mL, respectively. It was determined that neither of the hardaliye products had an inhibitory effect on other bacteria and yeast species (MIC: 250-1000 µg/mL) (Table 2).

Discussion

Grape is one of the widely grown fruits that has an economic role in the production of wine, fruit juice, jam and raisins, has rich phenolic components and has positive contributions to human health. Grape skin, pulp, juicy parts and seeds are rich in phenolic components. These components can be classified as phenolic acids, flavonoids, stilbenes. Phenolic compounds in fresh grapes and commercial grape juices can play an important role in preventing various diseases related to oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases, related to their antioxidant activity (1-3). Due to their antioxidant properties and abundance in the diet, phenolic compounds have become interesting to researchers and manufacturers.

In recent years, a discipline called "food-omics" has emerged that examines the fields of food and nutrition with the application and integration of advanced omic technologies to protect the health of consumers and ensure their trust (7). In this context, omic technologies such as genomic, epigenomic, transcriptomic, proteomics, metabolomics, metagenomics and have been accepted as the basic tools used in food-omics (8). The main purpose of metabolomics in the field of food-omics is to identify and quantify small (<1000-1500 Da) molecules (such as amino acids, lipids, carbohydrates, phenolic compounds, vitamins, organic acids, drugs) in food and nutrition studies. LC-HR/MS has been used in various metabolomic studies due to its wide dynamic range and reproducible quantitative analysis and performs well in profiling secondary metabolites (9, 10).

Data in the literature show that products with rich phenolic content reduce oxidative stress and the incidence of chronic diseases. Grape is one of the most important fruits in our diet due to its diversity in terms of phenolic components. With the antioxidant effect of these compounds, the consumption of products derived from grapes reduces the risk of cardiovascular disease. In addition, these components have been shown to have anti-cancer, anti-microbial and anti-inflammatory activities. The most common phenolic compounds found in grapes are in the group of flavonoids, phenolic acids and stilbenes. The most dominant class among the flavonoids found mostly in the core and skin part of the grape is flavan-3-ols, and this group includes compounds such as (+)-catechin, (-)-epicatechin, (-)-epigallocatechin. Another important group of flavonoids is flavanols, and compounds such as quercetin, myricetin, hyperoside (quercetin-3-O-galactoside) are included in this group. Phenolic acids, on the other hand, are mostly found in the skin and pulp of grapes. Phenolic compounds such as p-coumaric acid, caffeic acid are included in this group. Another phenolic component found in grape and grape products is resveratrol, which is in the class of stilbenes (1-3).

Studies on grape-derived products have shown that many factors can affect phenolic composition. Concentrations of (+)-catechin and (-)-epicatechin were determined as 500.52 ± 12.33 and 53.48 ± 19.78 mg/L, respectively, in grape juices obtained from red grapes of the genus *Vitis labrusca* L. produced by organic farming in Brazil. It has been reported that these data are higher than those obtained by traditional agriculture (79.89 ± 30.19, 14.40 ± 0.77 mg/L, respectively) (11). In the study conducted by Faikoğlu et al. in 2016, hardaliye was

produced from various grapes collected in the Tekirdağ region in the laboratory environment and the (-)-epicatechin concentration in Adakarası and Kalecik black grapes was determined as 21.89 ± 0.072 mg/100 mL and 20.55 ± 0.028 mg/100 mL, respectively (12). Silva et al. investigated the effect of different processing technologies such as "Hot press" (HP), "Cold press" (CP), "Hot break" (HB) and "Artisanal" used to produce grape juice on the content of phenolic components. The highest concentrations of (-)-epigallocatechin, (-)-epicatechin (17.23 mg/L, 50.30 mg/L, respectively) were detected in grape juices obtained with HB (13). In our study, (-)-epicatechin and (+)-catechin concentrations in H1 were 4.023 mg/L and 4.809 mg/L, respectively, while it was 9.428 mg/L and 5.381 mg/L in H2. The concentration of (-)-epigallocatechin was 7.108 mg/L in H1 and 2.5859 mg/L in H2. Compared to the data in the literature, the concentrations of (+)-catechin, (-)-epigallocatechin and (-)-epicatechin in hardaliye samples were observed to be quite low in our study.

Another group of flavonoids is flavanols. It is reported that compounds such as quercetin, myricetin, and hyperoside in this group have antioxidant, anti-inflammatory and anticarcinogenic properties (1-3). In the study conducted by Amoutzopulos et al. from our country, quercetin concentrations in hardaliye samples were reported as 65.5±0.37 mg/L (14). While the concentrations of myricetin were 7.99 ± 0.99, 6.98 ± 0.90 mg/L in organic and traditional red grape juices, respectively, this amount was quite low in white grape juices (1.85 ± 0.46 mg/L) obtained by traditional agriculture. In the same study, it was observed that the concentration of quercetin was 3.91 ± 0.08 mg/L in red grape juices obtained by organic farming, while it was 4.27 ± 0.54 mg/L in traditional cultivation (11). In the study reported by Balea et al. in 2020, hyperoside concentrations in the fresh and fermented pulp of *Vitis vinifera* L. Fetească neagră grapes grown in Romania were found to be 0.804 ± 0.06 mg/100 mL and 10.813 ± 0.18 mg/100 mL, respectively, and hyperoside could not be detected in Pinot Noir grapes (15). In our study, hyperoside, quercetin, and myricetin concentrations in H1 were 5.067 mg/L, 3.305 mg/L, and 1.689 mg/L, respectively, while in H2, it was found to be 0.034 mg/L, 0.798 mg/L, and 0.891 mg/L, respectively. It is lower than the data in the literature (Table 1). Caffeic acid and p-coumaric acid concentrations, which are among phenolic acids with antimicrobial, anticancer and anti-inflammatory activities, were reported as 60 ± 1.15 mg/L and 21 ± 1.71 mg/L, respectively, in the study conducted by Amoutzopulos et al. (14). In the study conducted by Toaldo et al. in 2015, the caffeic acid and p-coumaric concentrations (29.95 ± 1.57, 11.23 ± 0.16 mg/L, respectively) in red grape juices obtained by organic farming were compared to traditionally obtained red grape juices (14.08 ± 0.17, 10.73 ± 0.51 mg/L, respectively) was observed to be higher (11). In our study, while the concentration of p-coumaric acid is undetectable in H1, it is 31.87 mg/L in H2. Caffeic acid concentrations were found to be 0.312 and 1.559 mg/L in H1 and H2, respectively. It has been reported that resveratrol, which is in the stilbenes group among phenolic compounds, has a protective effect against many diseases, especially cancer and heart diseases (1-3). Trans-resveratrol concentration was determined as 2.72 ± 0.28 mg/L in the study reported by Amoutzopulos et al in 2013. The data of this study are similar to the resveratrol concentration (2070 ± 260 µg/L) detected in commercially available hardaliye products in the study reported by Ilıkkan et al. from Edirne in 2017 (14, 16). Resveratrol concentrations in hardaliye samples in our study were determined to be 44.57 mg/L in H1 and 16.64 mg/L in H2, and the determined amounts are considerably higher than the data in the literature. Considering the literature data, we think that the reason why the results we obtained in our study differ from the data in the literature is due to factors such as the type of grape used in the production of hardaliye, the geographical region where it is grown, the type of agriculture, harvest time, product processing conditions, and climatic conditions.

Phenolic compounds are known to have an important role in preventing various health problems such as cancer, cardiovascular and neurodegenerative diseases associated with

oxidative stress due to their antioxidant activity (1-3). Many studies have mentioned the relationship between phenolic compound composition and free radical scavenging activities. In a study reported from Brazil in 2013, the DPPH free radical scavenging activity of grape juices obtained from grapes harvested at different times of the year in 2010 was found to be 100%, and this ratio is showed high correlation with the anthocyanin content varying between 44.3 ± 2.01-129.5 ± 2.82 mg/100 mL (17). In the study reported by Nile et al. from Korea, they compared the phenolic content and antioxidant activity of grape skins and pulp in different grape varieties. While the antioxidant activity of extracts in grape peel was between 12.5% and 60.2%, this rate varied between 35.4% and 84.5% in extracts obtained from grape pulp. A statistically significant correlation was observed between the phenolic content obtained from the extracts and free radical scavenging activity (18). In the study conducted by Gündüz et al in 2019, DPPH free radical scavenging activity was reported to be between 80-90% in homemade hardaliye and 70-80% in commercial hardaliye, and the total phenolic content in hardaliye products was observed to be between 2029.30-2193.08 mg/L. In our study, the antioxidant activity in H1 and H2 was determined as 2.07±0,004% and 2.49±0,004% respectively. Compared to the data in the literature, the phenolic component contents and thus free radical scavenging activity of the hardaliye samples in our study are quite low. For this reason, it cannot be mentioned that the products in our study show antioxidant activity.

Ascorbic acid is thought to be a functional food ingredient, as it is an important bioactive compound found naturally in fruits and vegetables with antioxidant properties. For this reason, grapes and products derived from grapes also constitute an important part of the daily diet, as they contain varying concentrations of vitamin C (20). In 2013, Amoutzopulos et al. reported the vitamin C concentration in hardaliye products as 2.35 ± 0.07 mg/L (14). In a study conducted in Algeria in 2014, the ascorbic acid content of various grapes varied between 12.33 ± 0.01-30.80 ± 4.98 mg/100 mL (21). In our study, it was found as 21.295 mg/L in H1 and 26.84 mg/L in H2. Studies have reported that ascorbic acid content in fruit and vegetables may vary depending on factors such as climatic conditions (exposure to sunlight and weather), agricultural practices (fertilizers), crop maturity, harvest method, post-harvest processing conditions (storage), species, genotype (22, 23).

Fumaric acid is an organic acid that is used as an acidifier in beverages and is responsible for the sour taste (24). Studies have reported that mold species such as Rhizopus and Aspergillus produce fumaric acid (25). It has been observed that the addition of fumaric acid to foods and beverages causes bactericidal activity against foodborne pathogens (26). In a study reported in China in 2020, trace amounts of fumaric acid (0.002-2.18 mg/100 mL) were detected in grape juices obtained from different grape varieties (27). Similarly, in the study reported from Spain in 2021, trace amounts of fumaric acid were detected in the hydro-ethanol extracts of purple grape seeds (28). In our study, fumaric acid concentrations were seen as 59.55 and 224.522 mg/L in H1 and H2, respectively (Table 1). Although fumaric acid concentrations were higher in the samples analyzed in our study compared to these studies, the antibacterial activity could not be detected (Table 2).

When the literature data are reviewed, it has been reported that phenolic compounds show antimicrobial efficacy (1-3). Filocamo et al. showed that white grape juice extracts were effective against *S. aureu*s ATCC 6538P (MIC: 3.9 µg/mL), *S. epidermidis* ATCC 49134 and *S. epidermidis* ATCC 35984 (MIC: 15.62 µg/mL) strains in Italy (29). In the study conducted by Xu et al. in 2015, the highest ratio of catechin and epicatechin was found among the flavonoid components identified in the pulp obtained from grapes grown in Virginia. It has been reported that these fibers show antibacterial activity against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 29213 (30). In the study conducted by Gündüz et al., it was determined that homemade and commercial traditional hardaliye beverages produced from

grapes grown in our country showed antimicrobial activity on *S. aureus, B. cereus, Salmonella typhimurium* (MIC 4.53-150 mg/mL) (19). In a study conducted in 2021 in Spain, it was reported that extracts obtained from grape seeds of the Albariño genus *Vitis vinifera* had high concentrations of catechin and oligomers and that these extracts were effective against multiple resistant *S. aureus* strains (MIC: 5 mg/mL) (28). In our study, it was found that hardaliye samples showed antibacterial activity only on *S. epidermidis* ATCC 49461 strain (H1, MIC: 15.625 µg/mL; H2, MIC: <3.9 µg/mL), it was ineffective on other bacteria and *Candida* species (MIC: 250-1000 µg/mL) was determined. There is no study in the literature investigating the effectiveness of hardaliye on *H. pylori*. However, studies have reported that various grape extracts are effective on *H. pylori* (31). In our study, it was observed that hardaliye samples did not show antibacterial activity (MIC: 1000 µg/mL) on the strain of *H. pylori* ATCC 43504.

Conclusion

In the studies in the literature, it has been observed that the phenolic composition of grapes and products obtained from grapes may vary depending on the type of the grape, the geographical region where it is grown, climate, harvest and post-harvest processes. When grape juices obtained from grapes and grapes are evaluated in terms of antioxidant activity, free radical scavenging activity is compared with the amount of phenolic component. It was observed that the phenolic component concentrations determined in our study were generally lower than the literature findings. Therefore, the low DPPH free radical scavenging activity in hardaliye products was thought to be related to the low concentrations of phenolic components. To produce higher quality products and improve their biological activities on human health, it is necessary to support the factors affecting the phenolic composition in hardaliye production.

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Table 1. Phenolic components and their amounts in selected hardaliye samples

Chemical Name	**m/z***	**Ionization mode**	**H1 (mg/L)**	**H2 (mg/L)**	**U** (%)**
Ascorbic acid	175.025	Negative	21.295	26.84	3.94

(-)-Epigallocatechin	307.081	Positive	7.108	2.5859	3.09
(-)-Epigallocatechin gallate	459.092	Positive	0.479	<LOD	3.76
(+)-Catechin	289.072	Negative	4.809	5.381	3.31
Chlorogenic acid	353.088	Negative	0.48	0.575	3.58
Fumaric acid	115.004	Negative	59.55	224.562	2.88
(-)-Epicatechin	289.072	Negative	4.023	9.428	3.17
Verbascoside	623.198	Negative	0.024	0.053	2.93
Orientin	447.093	Negative	<LOD	0.129	3.74
Caffeic acid	179.035	Negative	0.312	1.559	3.06
(+)-trans taxifolin	303.051	Negative	<LOD	0.246	3.06
Naringin	579.172	Negative	<LOD	0.064	3.57
p-Coumaric acid	163.040	Negative	<LOD	31.87	3.79
Rosmarinic acid	359.077	Negative	<LOD	0.118	3.46
Hyperoside	463.088	Negative	5.067	0.034	2.86
Dihydrokaempferol	287.056	Negative	0.033	0.058	4.30
Ellagic acid	300.999	Negative	0.383	0.423	4.20
Quercitrin	447.093	Negative	0.271	0.001	3.78
Myricetin	317.030	Negative	1.689	0.891	4.18
Quercetin	301.035	Negative	3.305	0.798	2.95
Herniarin	177.055	Positive	0.251	0.082	3.89
Salicylic acid	137.024	Negative	0.626	0.696	1.89
Naringenin	271.061	Negative	0.052	0.032	4.20
Kaempferol	285.040	Negative	0.127	<LOD	3.56
3'-O-methyl quercetin	315.051	Negative	0.118	0.01	3.58

Apigenin	269.046	Negative	0.01	0.01	2.87
Chrysin	253.051	Negative	0.018	0.019	3.24
Resveratrol	229.085	Positive	44.57	16.64	3.17
***m/z:** mass to charge ratio, ****U:** measurement uncertainty					

Table 2. MIC results of hardaliye samples against in tested microorganisms

	Test material (µg/mL)	
Microorganisms	**H1**	**H2**
S. aureus ATCC 25923	1000	1000
E. coli ATCC 25922	1000	1000
P. aeruginosa ATCC 27853	1000	1000
E. faecalis ATCC 29212	500	500
A. baumannii ATCC 19606	1000	250
K. pneumoniae ATCC 70063	500	500
B. cereus ATCC 14579	500	500
S. epidermidis ATCC 49461	15.625	< 3.9
C. albicans ATCC 66027	1000	250
C. glabrata ATCC 2001	1000	250
H. pylori ATCC 43504	1000	1000

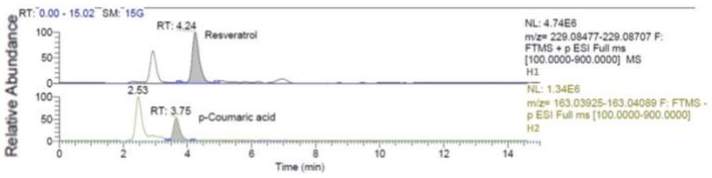

Figure 1. LC-HR/MS chromatograms of resveratrol and p-coumaric acid in H1 and H2

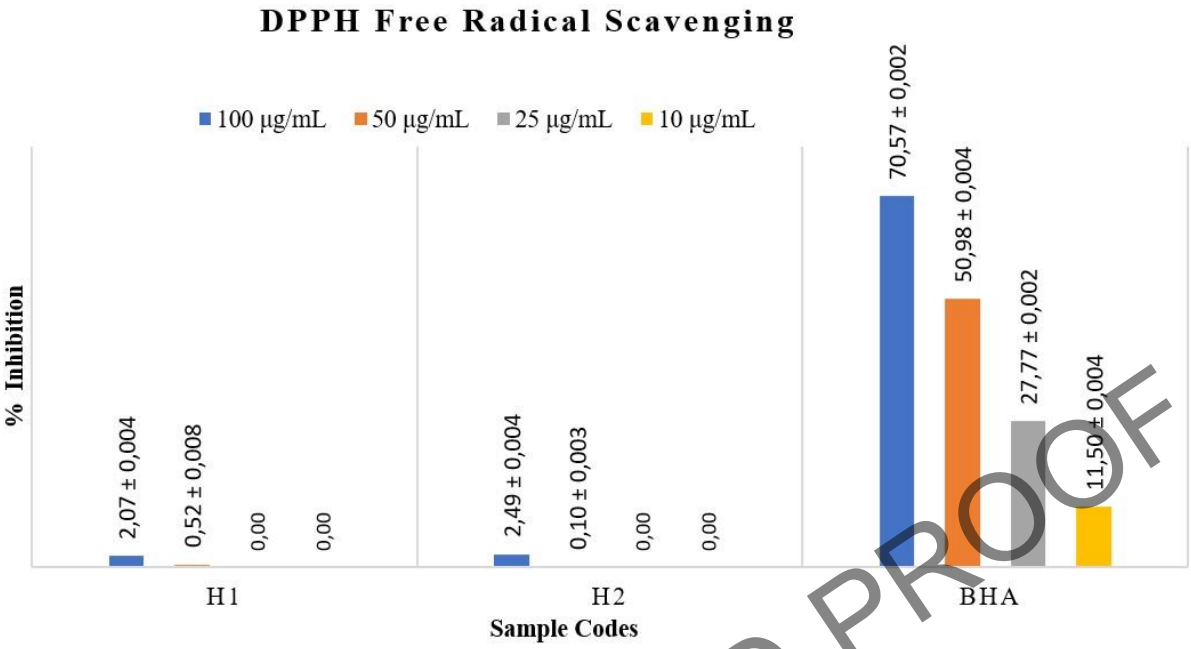

Figure 2. DPPH free radical scavenging activity results