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Production of Functional Chocolate with *Lycium Barbarum* L. (Wolfberry) Additive

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ABSTRACT

In this study, a new bitter chocolate with functional properties was produced by using different tempering temperatures with different ratios of wolfberry addition and physicochemical, antioxidant, textural and sensory properties of these chocolates were determined. It was observed that fruit ratios on bitter chocolate had a statistically significant (P<0.01) effect on DPPH, ABTS and hardness values, while there was no significant effect on total flavonoid amounts. It was determined that tempering temperature had a significant effect (P<0.01) on pH, reducing sugar, sucrose, total phenolic content (TPC), DPPH, ABTS, total flavonoids (TFC) and hardness values, while it had no significant effect on total dry matter and total sugar values. The rich total phenolic and antioxidant content of the added wolfberry fruit and its substitution for sugar improved the functional properties of the chocolate. As a result of the study, it was possible to introduce a value-added functional product with wolfberry additive to the market, thus pioneering the production of new products as well as the fresh use of the fruit.

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Introduction

In recent years, people's demand for functional foods has been increasing. Chocolate is one of the food products that are very popular worldwide and consumed with pleasure by most people. In addition to its unique taste, aroma and structure, it is stated to increase sensory pleasure and pleasant emotions in humans. Chocolate is basically a product obtained from cocoa beans, the fruit of the cocoa tree (*Theobroma cacao* L.). Originating from ancient times, chocolate consists of cocoa liquor, cocoa butter, sugar and milk. Chocolate can be produced in different types, mainly dark, milk and white (Aranguren and Marcovich, 2023).

Cacao, the main ingredient of chocolate, was first cultivated 5300 years ago in Central America by the Mayo-Chin chipe people. Cocoa beans are a rich source of polyphenols and flavonoids and contain 50-57% lipids. The main components of these lipids, also known as cocoa butter, are oleic, palmitic and stearic fatty acids. In addition to containing compounds with antioxidant properties such as polyphenols, chocolate and its products, which are important sources of carbohydrates and fats, also contain minerals such as potassium, magnesium, copper and iron. Today, there is an increasing interest in bitter chocolate, which is especially rich in polyphenol and flavonoid content. Basically, bitter chocolate is produced from sugar, cocoa butter and cocoa liquor. It is emphasized that the total polyphenol and flavonoid content of bitter chocolate is five times higher than milk and white chocolate. While polyphenols cause bitterness and astringency in cocoa beans, they are reported to be 5-6% in fresh cocoa beans. Flavonols in particular are responsible for bitterness in bitter chocolate and represent the flavor and organoleptic properties of bitter chocolate. Cocoa beans and cocoa contain flavonols, anthocyanin, procyanidins, stilbenes, phenolic acid, methylxanthine, caffeine and theobromine. Although bitter chocolate production varies depending on the product type, it basically consists of 6 steps: mixing, thinning, conching, tempering, shaping and cooling. The first step in chocolate processing is weighing and mixing the ingredients. Cocoa liquor, cocoa butter, sugar, milk powder and lecithin are thoroughly mixed at 40-50 °C for 12-15 minutes (Beckett, 2011).

Bitter chocolate production varies depending on the product type, but basically consists of 6 steps: mixing, thinning, conching, tempering, shaping and cooling. The first step in chocolate processing is weighing and mixing the ingredients. The second step in chocolate production, thinning, aims to reduce the particle size in the liquor to achieve a smooth structure. Following the mixing and thinning for the production of chocolate comes the conching stage. Because the conching process is the stage that affects the viscosity and texture of chocolate, it is one of the important steps that increase the quality of chocolate.

Conching can be defined as stirring the chocolate at 50°C for a long time. Tempering is defined as the pre-crystallization of cocoa butter at controlled temperatures to promote the crystallization of cocoa butter to give it a more stable poliform structure. Considering the positive and major effects of tempering on the quality characteristics of chocolate, it is possible to say that tempering is the most important production stage of chocolate. After tempering, the chocolate is shaped (molding). The product is then rapidly cooled to solidify the liquid phase mixture. Afterwards, the chocolates are removed from the mold by moving them from their corners with the help of force applied in the opposite direction (Şentürk, 2003). Bitter chocolate is known to have many positive effects on health. In particular, it is considered a functional food due to its anti-diabetic, anti-inflammatory and anti-microbial properties, as well as providing protection against cardiovascular diseases, various types of cancer, Alzheimer's, Parkinson's and many other brain-related diseases (Samanta et al. 2022).

Goji berry fruit (*Lycium barbarum* L.), also known as wolfberry, is widely consumed in China and Asian countries. Wolfberry also grows in the Mediterranean region and in temperate regions worldwide. While L. barbarum grows up to 3 m in height, L. chinense has a slightly smaller structure and its fruits are about 1-2 cm in size, depending on the species. China is the most important producer with an annual production of 95,000 tons. The wolfberry, which has specific colors ranging from yellow and red (L. *barbarum*) to black (L. *ruthenicum*), has a very pungent taste and flavor. Especially rich in vitamins and minerals, the primary fatty acid of wolfberry is linoleic acid, but it also contains fatty acids such as oleic, palmitic and stearic acids. The highest amino acids contained in the fruit are proline and serine. Wolfberry is known to be rich in bioactive compounds. It has also been reported to have many positive effects on human health such as antioxidant, anti-inflammatory, antimicrobial, immunostimulant, anti-diabetic. In addition, wolfberry fruit is utilized in various food processes including meat, dairy and cereal products (Masci et al. 2018).

In recent years, studies on goji berries have revealed many positive health effects. The increasing scientific evidence for the health-promoting effects of goji berries has increased interest in the possible application of goji berries or extracts as raw materials or functional additives in various food products. Many studies have been carried out to improve the functional properties of bitter chocolate and to obtain new products (Sik et al. 2021).

However, it is not possible to produce dark chocolate by applying different ratios of wolfberry and different production conditions. In this study, it is aimed to determine the effect of different ratios of goji berries (0% (control), 10%, 15%, 20%) and different production conditions (28°C, 30°C and 32°C) on the physical, chemical, antioxidant capacity and texture profile of dark chocolate and to produce a different product with functional properties that we have not encountered before in the literature. In this study, functional dark chocolate was produced using dried goji berry (*Lycium barbarum* L.) fruit instead of sugar. Every day, a new formulated functional product is added to the functional food caravan, which is becoming more and more important, and the functional food market is growing day by day. With this new product, it is expected to contribute to both healthy nutrition and the national economy. Based on the definition of functional food, it will be possible to introduce an important functional new product to the literature and the market with the active ingredients in goji berry fruit and chocolate in the content of this product.

Materials and Methods

Materials

In this study, dried wolfberry (*Lycium barbarum* L.) was obtained from Gojiberry Turkey brand, which is marketed as drug-free and natural from Antalya region, and 100% sugar-free and additive-free dark couverture was obtained from Aroha brand and used as research material.

Methods

Production of bitter chocolate with the addition of dried Wolfberry

Since the moisture content in the chocolate significantly affects the shelf life, dried wolfberries were used in the production. In order to ensure homogeneous distribution in the bitter chocolate, the wolfberries were frozen the day before and cut into small pieces as. Unsweetened bitter couverture was liquefied at 50ºC, then thickened and the liquid dark chocolate was tempered at 3 different temperatures (28 ºC, 30ºC, 32ºC). It was transferred to molds to be shaped and wolfberries were added by adjusting the fruit ratios (0% (control), 10%, 15%, 20%). The molds were cooled at +4ºC ambient temperature.

Total dry matter (TDM) analysis

The dry matter content was calculated by taking 3 g each of the dark chocolate samples dried in an oven at 105ºC, cooled and tared in drying containers and the process was continued until it reached a constant weight and then the dry matter content was calculated (Cemeroğlu, 2007).

Ash analysis

The crucibles dried in the muffle furnace were tared, then 3 g of sample was added and the burning process was continued at 550±50ºC until white ash was formed. The amount of ash was determined by calculating the samples cooled in the desiccator (Cemeroğlu, 2007).

pH analysis

Bitter chocolates were weighed approximately 3 g and minced finely. It was diluted with pure water and homogenized with the help of ultraturrax. The diluted and filtered dark chocolate samples were measured by immersing the pH meter probe (Cemeroğlu, 2007).

Determination of total sugar, sucrose, reducing sugar

Approximately 18 g of the fruit-added chocolate samples, whose total sugar content was estimated by preliminary trials, were homogenized and prepared for analysis. Approximately 50 g of the control chocolates were taken and prepared for analysis. Pure water heated to 65ºC was added to the samples. They were then cooled. Lead acetate solution was added and sodium oxalate was added to the samples taken in appropriate volumes in a balloon jug and waited for about 10 minutes. 50 ml sample was taken and the mixture was completed to 250 mL and ready to be used for invert sugar content. A 50% HCl solution was added to the mixture and the samples kept in a water bath adjusted to 67 ºC were titrated with 5 mL Fehling A, 5 mL Fehling B, 25 mL pure water. Upon boiling, titration with methylene blue was performed. Afterwards, reducing sugar and total sugar amounts were calculated according to the amount of volume used (Cemeroğlu, 2007).

Preparation of Samples for (TPC), DPPH, ABTS, (TFC) Analysis

Approximately 2 g of sample was weighed, 20 mL of methanol/water (80/20%) was added and homogenized with the help of Ultraturrax at 20000 rpm for 5 min. It was then kept in an ultrasonic water bath for 30 min. Centrifuged at 6000 rpm for 15 min with the help of a cooled centrifuge. It was passed through Whatman (No:42) filter paper. Since chocolate is an oily product, it was degreased with n- hexane and a preliminary study without hexane was performed. This extract was used for total phenolic content, DPPH, ABTS and total flavonoid content analysis.

TPC determination

Fruit-added chocolate samples and 0.1 mL of the extracts prepared for TPC analysis with the control were precisely transferred to test tubes. Then 800 µL of 0.2 N Folin-Ciocalteu and 800 µL of 10% Na_2CO_3 solution were added respectively. The chocolate samples were made up to 2.5 ml with distilled water and incubated for 30 minutes and measured at 760 nm absorbance value. The amount of TPC was calculated in mg GAE/g sample with the gallic acid standard curve prepared daily (Binici et al., 2021).

DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity determination

For chemical analysis, 39 mg of DPPH radical was taken and placed in a 100 mL glass flask. Then, 0.3 mL of the chemical solution completed with ethyl alcohol and sample extracts were added to 100 mL and completed with methanol to 2 mL. The test tubes were mixed in a vortex. Then the samples stored in the dark were measured at 517 nm absorbance value. IC_{50} values were calculated based on the absorbance values obtained (Binici et al., 2021).

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ABTS radical was mixed with ABTS solution prepared at a concentration of 2 mM with distilled water and 2.45 nM potassium persulfate solution. The prepared chemical was kept in the dark. Before analysis, the absorbance of the solution was diluted to 700-800 at 734 nm. After the extracts were added to the test tubes in different µg/µL amounts, ABTS solution was transferred to a total volume of 2 mL. Each tube was mixed in a vortex and incubated in the dark for half an hour. The samples were measured at 734 nm absorbance. The color lightening in the sample solutions is an indicator of the antioxidant effect and the % inhibition values of the samples at different concentrations were calculated as IC50 value (concentration inhibiting 50% of the radical) using the following equation (Köksal et al. 2009).

Total flavonoid content (TFC) determination

In a modified determination according to Binici et al. (2021), 0.250 mL of the samples were taken and 0.075 mL of $NaNO_2$ (5%) solution was added. The chemical mixture was allowed to stand. 0.150 mL of $AlCl_3$ (10%) solution was added to the mixture. The mixture was allowed to stand again and then 0.500 mL of NaOH (4%) solution was added and completed with 2.5 mL of distilled water. The samples were measured at 510 nm absorbance. The amount of TFC was calculated in µg QE/g sample using a Quercetin standard curve prepared daily.

Color analysis

Color analysis of the chocolate samples was performed by colorimeter (Chroma Meter, CR-5). As a result of the measurement, L^*, a^* and b^* values were used to examine the color of wolfberry fruit and dark chocolate produced in terms of physical parameters (Youssef and Mokhtar 2014).

Texture analysis

Texture determination was performed by drilling with TA.XT-plus Analyser (device) as shown in Figure 1. In the analysis, measurements were made from 4 points with P/2 cylinder probe in 2 parallel. Texture measurement parameters modified according to Ali et al. (2001) were pre-test speed 2.00 mm/sec, test speed 2.00 mm/sec, distance 5mm, trigger force 5.0 g, test time approximately 3 minutes.

Sensory analysis

Sensory evaluation of the chocolate samples with wolfberry addition was carried out by 10 trained panelists. The trained panelists evaluated 12 types of bitter chocolate in two stages at different times according to appearance (color, gloss, surface), texture, chewiness, odor, taste and general acceptability criteria, which were defined randomly out of 5 points.

Statistical Analysis

The research was carried out with 4 different fruit ratios (0% (control), 10%, 15%, 20%) bitter chocolate and 3 tempering temperatures in a 4×3 factorial arrangement according to a complete chance experiment plan. The data extracted from the research results were analyzed by analysis of variance with SPSS program. Sources of variation were evaluated by Duncan Multiple Comparison Test at 95% reliability level.

Figure 1. Texture determination of bitter chocolate with wolfberry addition

Results and Discussion

Physical and Chemical Properties of Wolfberry

The results of physical and chemical analysis of the wolfberry fruit used in the study are given in (Table 1). In the literature, the number of studies supporting the results we found is quite high. However, there are also studies with different results. In particular, these differences are due to the climatic and geographical conditions in which the wolfberry fruit grows (Pedro et al. 2019).

Total dry matter content of dried wolfberry fruit was 84% and total ash content was 3.69%. Talay et al. (2021) reported that the dry matter content of dried wolfberry fruit was 89.40% and ash content was 4.30%. The dry matter contents of the fruit increased according to the drying rate and similar results were reported.

The pH value of dried wolfberry fruit was determined as 5.68. Çolak et al. (2016) reported the pH value of fresh wolfberry fruit as 3.25- 4.36. Şengün and Kadakal (2021) determined the pH value of ripe wolfberry fruit as 5.21 and reported that the pH value increases as the fruit ripens. The pH of the dried fruit used in our study was slightly higher than other studies. This suggests that the fruit was dried after reaching full maturity.

Total sugar, reducing sugar and sucrose content of dried wolfberries were determined as 61.17 g/100 g, 49.73 g/100 g and 11.44 g/100 g, respectively. Talay et al. (2021) determined 59.26%, 57.35% and 1.90%, respectively. Batu and Kadakal (2021) determined the fructose, glucose and sucrose content of wolfberry dried at 70°C as 17.22 g/kg, 17.55 g/kg and 0.016 g/kg, respectively. In our research on dark chocolate with wolfberry, the sugar content of the dried fruit we used was higher than other studies. This may be due to factors such as the type of fruit we use, growing conditions, climate, full ripening and adequacy of the drying process. Conscious consumers have turned to sugar-free or low-calorie foods with increased functionality for a healthier life. In the bitter chocolates we produced in order to provide functional properties, the production was made without sugar and it was aimed to ensure that the sensory properties of the chocolate were at an acceptable level with only the sugar of the fruit. The high sugar content of the dried wolfberry fruit, which we used as a sugar substitute, is in accordance with the purpose of the study. With the high sugar content of the fruit, the taste that consumers are used to is met with natural production, while other components contained in the fruit contribute to the production of functional chocolate by increasing the functionality of chocolate.

The total phenolic content of dried wolfberry was determined as 51.93 µg GAE/g. Islam et al. (2017) reported the total phenolic content of wolfberry fruit as 2.17-9.01 mg GAE/g. Talay et al. (2021) 9.05 µg GAE/g; He et al. (2018) 17.92 mg GAE/100 g. The total phenolic content of the fruit gave similar results with other studies.

Table 1. Physical and Chemical Properties of Dried Wolfberry Fruit

Analysis	Wolfberry
Total Dry Matter (%)	84.00±0.80
Total Ash (%)	3.69±0.06
pH	5.68±0.04
Total Sugar (g/100g)	61.17±0.19
Reducing Sugar (g/100g)	49.73±0.28
Sucrose (%)	11.44±0.47
TPC (µg GAE/g)	51.93±0.73
DPPH IC_{50} (µg/mL)	1804.84±39.47
ABTS IC_{50} (µg/mL)	372.83±22.15
TFC (µg QE/g)	4.77±0.03
*L**	28.09±0.97
*a**	19.99±2.45
*b**	11.25±0.46

Abbreviations: QE: Quercetin equivalence

Table 2. TDM, total ash and pH values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28°C	30°C	32°C
TDM (%)	Control	98.07±0.14^{a}	98.20±0.24^{a}	98.09±0.13^{a}
	10	97.23±0.61^{b}	96.66±0.79^{b}	96.31±0.49^{b}
	15	96.42±0.28^{c}	96.11±0.12^{b}	95.47±0.09^{c}
	20	95.46±0.91^{d}	95.76±1.30^{c}	95.28±0.33^{c}
Total ash (%)	Control	4.92±0.18^{a}	4.77±0.04^{a}	4.61±0.05^{a}
	10	4.47±0.06^{c}	4.68±0.09^{b}	4.66±0.08^{a}
	15	4.59±0.17^{b}	4.59±0.05^{c}	4.45±0.02^{c}
	20	4.30±0.08^{d}	4.39±0.11^{d}	4.53±0.35^{b}
pH	Control	6.54±0.06^{a}	6.60±0.01^{a}	6.62±0.01^{a}
	10	6.42±0.04^{c}	6.47±0.01^{d}	6.32±0.01^{c}
	15	6.32±0.02^{d}	6.50±0.01^{c}	6.39±0.01^{b}
	20	6.45±0.03^{b}	6.53±0.01^{b}	6.29±0.01^{d}

Different letters (a-d) in the same column are significantly different (P<0.01).

Table 3. Total sugar, reducing sugar and sucrose values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28°C	30°C	32°C
Total sugar (g/100g)	Control	nd	nd	nd
	10	6.12±0.02^{c}	6.14±0.02^{c}	6.13±0.02^{c}
	15	9.18±0.03^{b}	9.19±0.03^{b}	9.18±0.03^{b}
	20	12.22±0.00^{a}	12.25±0.03^{a}	12.26±0.04^{a}
Reducing sugar (g/100g)	Control	nd	nd	nd
	10	5.06±0.00^{c}	5.58±0.01^{c}	5.44±0.01^{c}
	15	8.15±0.02^{b}	8.45±0.02^{b}	8.65±0.02^{b}
	20	11.60±0.03^{a}	11.71±0.00^{a}	11.58±0.03^{a}
Sucrose (g/100g)	Control	nd	nd	nd
	10	1.06±0.02^{a}	0.56±0.03^{b}	0.69±0.03^{a}
	15	1.03±0.01^{a}	0.74±0.00^{a}	0.53±0.00^{b}
	20	0.63±0.03^{b}	0.53±0.03^{b}	0.67±0.00^{a}

Different letters (a-c) in the same column are significantly different (P<0.01). Abbreviations: nd: not determined.

The IC_{50} value determined for DPPH* radical of dried wolfberry was 1804.84 µg/mL. Islam et al. (2017) reported the DPPH* value as 16.07-35.86 µmol TE/g. Soares et al. (2018) reported the IC50 value determined for DPPH* radical as 2.48 mg/mL. DPPH* content of the fruit gave similar results with other studies.

The IC_{50} value for ABTS* content of dried wolfberry was determined as 372.83 µg/mL. Islam et al. (2017) reported the ABTS content of wolfberry as 53.92-180.03 (µmol TE/g); Soares et al. (2018) reported it as 10.67 µmol Trolox/g. The ABTS* content of the fruit was higher than other studies. It is thought that the high antioxidant capacity will have a positive effect on the functionality of bitter chocolate with wolfberry.

The TFC of dried wolfberry was determined as 4.77 µg QE/g (Table 3). Islam et al. (2017) determined the total flavonoid content of wolfberry as 2.67-12.32 mg QE/g. According to the studies, the flavonoid content of the fruit was high.

The *L** value of dried wolfberry fruit was 28.09; *a** value was 19.99; *b** value was 11.25 (Table 1). Batu and Kadakal (2021) reported that the L value of fresh and dried up to 70°C wolfberries varied between 25.97- 21.99; *a** value 25.16- 14.67; *b** value 17.30- 9.62. It is expected that wolfberry, which has certain colors from yellow and red (*L. barbarum*) to black (L. ruthenicum), will differ in terms of color parameters.

Physical and Chemical Properties of Wolfberry Chocolates

Total dry matter, total ash and pH values of the control group and the bitter chocolates prepared with different ratios of wolfberry and tempered at different temperatures are given in (Table 2). While the total dry matter ratios of the control group and the chocolates containing different ratios of wolfberry ranged between 95.28-98.20%, the effect of fruit ratio on the dry matter content was found to be significant (P<0.01) and the effect of different tempering temperatures was not significant (Table 2). This is because high moisture in chocolate products causes a grainy and flaky structure, which is not desirable in terms of quality criteria (Shiehzadeh, 2019).

While the total ash ratios of the control group and chocolates containing different ratios of wolfberry ratios varied between 4.30-4.92%, the effect of fruit ratio on total ash ratio was found to be significant (P<0.01), while the effect of different tempering temperatures was not significant. Peker (2011) reported that the ash ratios of bitter chocolate samples were between 1.82% and 1.87%. Total ash content decreased in the samples to which goji bery was added may be due to the decrease in total dry matter content. While the pH values of the control group and chocolates containing different ratios of wolfberry ranged between 6.29-6.62, it was determined that different tempering temperatures and fruit ratio had a significant (P<0.01) effect on the pH value of the samples. In terms of pH, it is understood that the control group and the chocolate samples in which fruit is used are different from each other, but the samples in which 10%, 15% and 20% fruit is used show similar characteristics. It was determined that three different tempering temperatures had a significant (P<0.01) effect on the pH value. Artık (1988) reported that heat treatment will cause a change in the pH of the fruit, although it is dependent on the duration and degree of heat treatment. The change in pH values of the samples is expected.

Total sugar, reducing sugar and sucrose contents of the control group and bitter chocolates prepared with different ratios of wolfberry and tempered at different temperatures are given in Table 3. While the total sugar values of the control group and the chocolates containing different ratios of wolfberry ranged between nd- 12.26 g/100 g, the effect of fruit ratio on total sugar content was found to be significant (P<0.01), while the effect of different tempering temperatures was not significant. In this study, wolfberry fruit used in the production of bitter chocolate affected the total sugar content of the chocolates (P<0.01). It is understood that chocolate samples differed from each other in terms of total sugar. Because sugar was not detected in the control group (0%) chocolates produced without the addition of fruit. However, Belščak-Cvitanović et al. (2015) determined that the total sugar content of chocolates varied between 91.27 and 451.17 mg/g. In another study by Cai et al. (2018), polysaccharides in wolfberry were found to have a protective effect on Type 2 diabetics. Although wolfberry fruit added to our samples at different ratios contains less sugar than other studies in terms of total sugar content, we expect the fruit to have a protective effect for Type 2 diabetes and to make a positive contribution to increase the functionality of the production.

While the reducing sugar content of the control group and chocolates containing different ratios of wolfberry ranged between nd- 11.71 g/100 g, it was determined that the effect of fruit ratio and different tempering temperatures on the reducing sugar content was significant (P<0.01). Reducing sugar was not detected in the control group (0%) chocolates produced without adding fruit. Shiehzadeh (2019) reported that the total amount of reducing sugars varied between 31.53 and 55.31 g/100g in his study on spreadable chocolate samples. Compared to other studies, the amount of reducing sugars in our samples was low. Considering the trend towards reduced calorie foods for healthy living, our samples are expected to attract interest. While the sucrose amounts of the control group and chocolates containing different ratios of wolfberry ranged between nd - 1.06%, it was determined that the effect of fruit ratio and different tempering temperatures on sucrose value was significant (P<0.01). Pirouzian et al. (2016) reported that sucrose-free chocolates were accepted by expert evaluators in their study using sugar alcohols instead of sucrose replacement. Aidoo et al. (2014) reported that they produced acceptable chocolate in terms of some physical and chemical quality characteristics in their study for sugar-free chocolate production. In the present study, sucrose values of our samples were low. Because sucrose was not detected in the control group (0%) chocolates produced without adding fruit. In diseases such as diabetes and obesity, foods with reduced calories should be preferred. This situation has revealed the need for new formulation chocolate researches that can provide functional chocolate properties by substituting sugar-free or sugar substitutes in chocolates that are consumed fondly.

The TPC, DPPH, ABTS and TFC of the control group and bitter chocolates prepared by adding wolfberry at different ratios and tempered at different temperatures are given in (Table 4). Vertuani et al. (2014) determined the amount of total phenolic content ranging between 16.28-38.13 mg GAE/g in their study. Godočiková et al. (2017) reported that the TPC content of bitter chocolate enriched with sea buckthorn and mulberry varied between 7.32-8.83 mg GAE/g. Considering the tendency of the researches to strengthen or enrich the chocolate content with functional structures such as phenolic substances and antioxidants, the fact that the TPC content of our samples is higher than the other research results is in line with our purpose.

While the IC_{50} value determined for DPPH radical scavenging activity of the control group and chocolates containing different ratios of wolfberry ranged between 301.53- 514.97 (µg/mL), it was determined that the effect of fruit ratio and different tempering temperatures on DPPH radical scavenging activity amounts was significant (P<0.01). Vertuani et al. (2014) determined the IC_{50} value determined for DPPH* radical at levels ranging between 0.55-0.81 mg/mL in their study. Godočiková et al. (2017) reported that the IC_{50} value of bitter chocolate enriched with sea buckthorn and mulberry for DPPH* radical ranged between 3.64-4.39 mg/mL. In our samples, the highest DPPH radical scavenging activity was found in bitter chocolate samples with 20% fruit ratio, but the activity was not directly proportional to the fruit ratio. This may be due to the application of the method. While the ABTS IC_{50} values of the control group and chocolates containing different ratios of wolfberry ranged between 164.05-411.73 µg/mL, it was determined that the effect of fruit ratio and different tempering temperatures on ABTS IC_{50} values was significant (P<0.01). Martini et al. (2018) found the amount of ABTS in dark chocolate samples between 1.8-10 mmol TE/100 g. The results showed that different fruits had diverse antioxidant capacities and the variation was very large (Fu et al., 2011). The higher ABTS content in our samples compared to the results of other studies may be due to the applied method and fruit ratios. While the TFC values of the control group and chocolates containing different ratios of wolfberry ranged between 8.11-14.94 µg QE/g, the effect of tempering temperatures on total flavonoid amount was found to be significant (P<0.01), while the effect of different fruit ratio was not significant. Godočiková et al. (2017) reported 0.52-1.17 mg QE/g in another study on bitter chocolate. Flavonoids, which have an important share among phenolic compounds, are especially involved in the improvement of cardiovascular diseases. Scientific studies have been increased in foods containing flavonoids in terms of flavonoid consumption to protect and improve human health.

Table 4. TPC, DPPH, ABTS and total flavonoid content values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28°C	30°C	32°C
TPC (mg GAE/g)	Control	59.16±1.45^{b}	36.19±0.38^{d}	49.73±1.03^{c}
	10	44.64±2.04^{d}	56.78±0.10^{b}	63.18±0.57^{b}
	15	56.17±0.36^{c}	58.27±0.06^{a}	67.25±0.42^{a}
	20	61.79±2.35^{a}	49.63±2.03^{c}	68.50±0.06^{a}
DPPH• (IC_{50} µg/mL)	Control	301.53±0.78^{d}	514.97±5.47^{a}	464.13±4.80^{a}
	10	315.86±0.63^{c}	477.66±2.39^{d}	446.98±0.30^{b}
	15	416.45±0.67^{a}	486.21±3.53^{c}	383.83±2.60^{d}
	20	322.32±3.61^{b}	495.27±1.64^{b}	378.77±0.77^{c}
ABTS• (IC_{50} µg/mL)	Control	185.56±1.46^{c}	329.69±12.93^{c}	411.73±11.82^{a}
	10	202.41±7.77^{b}	397.08±7.12^{a}	329.56±11.79^{b}
	15	249.44±3.81^{a}	270.83±20.95^{d}	257.79±4.52^{d}
	20	164.05±12.38^{d}	336.72±4.19^{b}	280.46±1.88^{c}
TFC (µg QE/g)	Control	14.94±0.28^{a}	12.59±0.49^{a}	12.26±0.54^{b}
	10	14.30±0.07^{b}	11.89±0.12^{b}	12.02±2.96^{c}
	15	14.02±0.34^{c}	10.31±0.01^{c}	14.07±0.00^{a}
	20	13.64±0.08^{d}	8.11±0.03^{d}	14.04±0.09^{a}

Different letters (a-d) in the same column are significantly different (P<0.01). Abbreviations: nd: not determined. QE: Quercetin equivalence

Table 5. Color (*L**, *a** and *b**) values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28 °C	30 °C	32 °C
*L**	Control	31.77±0.07^{b}	32.51±0.06^{b}	31.82±0.33^{ab}
	10	31.35±0.12^{c}	31.59±0.13^{ab}	32.12±0.07^{b}
	15	31.98±0.02^{a}	31.56±0.07^{ab}	32.33±0.07^{a}
	20	32.02±0.19^{d}	31.65±0.04^{a}	31.68±0.04^{ab}
*a**	Control	2.90±0.06^{ab}	3.13±0.37^{a}	3.05±0.09^{a}
	10	2.96±0.13^{b}	2.83±0.02^{ab}	2.81±0.08^{ab}
	15	2.85±0.03^{ab}	2.76±0.02^{b}	2.71±0.08^{b}
	20	3.07±0.09^{a}	2.91±0.03^{ab}	2.91±0.04^{ab}
*b**	Control	2.03±0.08^{ab}	2.01±0.17^{ab}	2.33±0.17^{a}
	10	2.25±0.21^{b}	2.12±0.06^{a}	1.91±0.09^{c}
	15	2.09±0.01^{ab}	1.94±0.13^{b}	1.81±0.03^{d}
	20	2.62±0.11^{a}	2.00±0.03^{ab}	2.06±0.02^{b}

Different letters (a-c) in the same column are significantly different (P<0.01)

Table 6. Texture (Hardness) values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28°C	30°C	32°C
Hardness (N)	Control	27.80±1.50^{b}	27.64±0.36^{a}	24.02±1.05^{b}
	10	29.04±0.13^{a}	26.85±0.07^{b}	31.50±1.56^{a}
	15	18.53±0.19^{d}	15.71±0.04^{c}	21.83±0.01^{c}
	20	23.41±1.60^{c}	14.29±0.62^{d}	13.74±0.16^{d}

Different letters (a-d) in the same column are significantly different (P<0.01)

The color (*L**, *a** and *b**) values of the control group and bitter chocolates prepared by adding wolfberry at different ratios and tempered at different temperatures are given in (Table 5). While the *L** values of the control group and the chocolates containing different ratios of wolfberry ranged between 31.35-32.51, it was determined that the effect of fruit ratio on *L** value was significant at (P<0.05) level and the effect of tempering temperature was not significant. *L** value is one of the parameters indicating the lightness criterion of the samples (Gürsoy, 2021). Shiehzadeh (2019) reported that the *L** values of the chocolates they produced using different ingredients varied between 30.36 and 36.80, and Gürsoy (2021) reported that the *L** value of the samples varied between 21.64 and 26.91 in his research on bitter chocolate. In another study on milk and bitter chocolate, the average *L** value was determined as 25.8 (Yücekutlu, 2015). The *L** value of the present study is acceptable for the consumers.

While the *a** values of the control group and the chocolates containing different ratios of wolfberry ranged between 2.71-3.13, it was determined that the tempering temperature had no significant effect, while the fruit ratio had a significant effect on the *a** value at (P<0.05) level. Yücekutlu (2015) determined that the average *a** value in bitter chocolate samples was 4.3. Shıehzadeh (2019) reported that the *a** value varied between 10.84 and 11.80 in chocolate samples. Gürsoy (2021) determined that the *a** value of bitter chocolates varied between 13.19-15.14 in his study. While the *b** values of the control group and chocolates containing different ratios of wolfberry ranged between 1.81-2.62, it was determined that the effect of fruit ratio and tempering temperature on *b** value was significant (P<0.01). Shıehzadeh (2019) reported that the *b** value varied between 3.85 and 12.74 in his study on chocolate samples.

While the hardness values of the control group and chocolates containing different ratios of wolfberry ranged between 13.74-31.50 N, the effect of fruit ratio and different tempering temperatures on the hardness value was found to be significant (P<0.01) (Table 6). In this study, the effect of wolfberry fruit used in bitter chocolate production on the hardness value was P<0.01 significant. In terms of hardness, it was determined that the control group and the chocolate samples in which fruit was used were different from each other. The highest hardness value was determined in the samples to which 10% fruit was added, while the lowest hardness value was determined in the samples to which 20% fruit was added. It was determined that three different tempering temperatures had a significant (P<0.01) effect on the hardness value. It is seen that tempering chocolate samples at 30°C is more acceptable in terms of hardness.

The sensory analysis values of the control group and the bitter chocolates prepared by adding different ratios of wolfberries and tempered at different temperatures are given in (Table 7). Sensory evaluation of the bitter chocolates produced in the study was carried out by trained panelists at Atatürk University, Department of Food Engineering. Appearance, texture, chewiness, odor, taste and general acceptability values of the control group and chocolates containing different ratios of wolfberry were evaluated out of the highest 5 points and it was determined that they varied between 3.70 - 4.40, 3.70 - 4.30, 3.20 - 4.10, 3.50 - 4.30, 2.30 - 4.00 and 3.10 - 3.90, respectively. In terms of appearance, texture, chewiness and odor, the samples tempered at 32 °C with 20% fruit content were the most liked, while the samples tempered at 30 °C with 20% fruit content were the most liked in terms of taste.

Table 7. Sensory analysis (appearance, texture, chewiness, odor, taste, general acceptability) values of bitter chocolates prepared with different proportions of wolfberries and tempered at different temperatures

	Wolfberry ratios (%)	Tempering temperature (°C)		
		28 °C	30 °C	32 °C
Appearance	Control	3.70±0.82^{b}	3.80±0.79^{c}	4.00±1.05^{c}
	10	4.00±0.82^{a}	4.00±0.82^{b}	4.20±0.92^{b}
	15	4.00±0.82^{a}	4.20±0.79^{a}	4.00±0.94^{c}
	20	4.00±1.05^{a}	4.20±0.79^{a}	4.40±0.52^{a}
Texture	Control	3.80±1.03^{c}	3.70±0.68^{c}	4.00±0.94^{c}
	10	3.90±0.74^{b}	3.90±0.88^{b}	4.10±0.88^{b}
	15	3.70±0.95^{d}	4.20±1.03^{a}	3.80±0.92^{d}
	20	4.10±1.00^{a}	4.20±0.92^{a}	4.30±0.68^{a}
Chewiness	Control	3.20±0.92^{d}	3.40±0.97^{c}	3.60±0.70^{b}
	10	3.60±0.70^{c}	3.40±0.70^{c}	3.60±0.70^{b}
	15	3.50±1.08^{b}	3.80±1.14^{b}	3.50±0.97^{c}
	20	4.00±0.82^{a}	4.00±0.94^{a}	4.10±0.74^{a}
Odor	Control	3.90±1.00^{b}	3.80±0.79^{c}	3.70±0.95^{d}
	10	3.50±1.18^{d}	3.80±0.92^{c}	3.90±1.00^{c}
	15	3.80±0.92^{c}	4.10±0.88^{a}	4.20±0.79^{b}
	20	4.20±0.79^{a}	4.00±1.05^{b}	4.30±0.82^{a}
Taste	Control	3.80±0.92^{a}	2.30±0.95^{d}	3.60±0.52^{b}
	10	3.00±0.82^{c}	3.50±0.71^{c}	3.20±0.63^{c}
	15	3.50±0.97^{b}	3.90±0.88^{b}	3.60±0.84^{b}
	20	3.80±0.92^{a}	4.00±0.82^{a}	3.80±0.79^{a}
General Acceptability	Control	3.60±0.52^{b}	3.60±0.52^{c}	3.50±0.53^{c}
	10	3.10±0.74^{c}	3.40±0.52^{d}	3.50±0.53^{c}
	15	3.60±0.84^{b}	3.70±0.82^{b}	3.60±0.52^{b}
	20	3.90±0.88^{a}	3.90±0.88^{a}	3.90±0.74^{a}

Different letters (a-d) in the same column are significantly different (P<0.01)

Conclusions

Currently, the importance that people attach to nutrition is increasing. Chocolate is constantly consumed due to its sensory properties. Especially the conscious consumers' tendency towards low-calorie and functional chocolates is increasing day by day. Bitter chocolate is preferred more than other chocolates with its functionality and has been a product that has been experimented with new formulations. In the literature, it is seen that the number of studies on the enrichment of the composition of bitter chocolate is increasing. The main topics of the studies are adding antioxidant substances, reducing the calorie content, increasing the fiber content and alternative methods in production. In this study, 12 types of wolfberry bitter chocolate were produced by applying 4 different fruit ratios and 3 different tempering temperatures. It was determined that the added fruit ratio had a statistically significant effect on dry matter, total ash, pH, total sugar, reducing sugar, sucrose, total phenolic content, DPPH, ABTS, b^* and hardness of dark chocolates at p<0.01 level, while it had no significant effect on total flavonoid. Tempering temperature had a statistically significant effect on pH, reducing sugar, sucrose, total phenolic content, DPPH, ABTS, total flavonoids, b^* and hardness values of dark chocolates at p<0.01 level, while it had no significant effect on dry matter, ash, total sugar, L^* and a^*. In terms of sensory properties, the sample tempered at 32 °C with 20% fruit ratio was most liked and accepted by trained panellists. In addition, as expected, the highest antioxidant activity was determined in the samples with 15% and 20% fruit ratio of wolfberry, while the highest total flavonoid content was determined in the control group. As a result, it was concluded that the chocolate sample containing 20% fruit and tempered at 32°C was suitable for commercial production in terms of both physical, chemical and functional properties and the scores obtained in sensory evaluations.

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Author Contributions

Conceptualization, İ.G.Ş.; methodology, İ.G.Ş., H.İ.B., and E.A.; validation, İ.G.Ş.; investigation, H.İ.B., and E.A.; writing original draft preparation, H.İ.B., and E.A.; writing review and editing, İ.G.Ş.; supervision, İ.G.Ş. All the authors have read and agreed to the published version of the manuscript.

The Declaration of Conflıcts of Interest

There are no conflicts to declare.

The Declaration of Ethics Committee Approval

Ethics committee permission is not required for this study.

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