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# The Health Benefits of Jeju Gamgyul (*Citrus unshiu* Marc.) Brandy and Wine

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**Abstract** Beneficial health effects of fruits and vegetables in the diet have been attributed to the reduction of risk for some diseases, such as diabetes, chronic diseases, cancer, or cardiovascular diseases. Citrus and its peels have been used in Asian folk medicine due to abundant flavonoids and the fact that their pulp are usually consumed like juice or jam, although their functional effects are much higher than those of fruit peels. This investigation inquired into the effects of Gamgyul wine, which is derived from whole fruits of Citrus unshiu, with respect to antioxidant property, anti-diabetic activity, low density lipoprotein (LDL) oxidative inhibition and flavanones. In particular, antioxidant and antidiabetic activities of both Gamgyul wine and brandy were measured. However, the differences of the antioxidant and antidiabetic effects of Gamgyul wine were trifling between dynamite and microfiltration processes. In particular, the effect of antidiabetic activity was rather significant as demonstrated in the Gamgyul brandy. The results showed that α-glucosidase inhibition rates at 20 µL/mL for Gamgyul brandy and wine were 50% and 20%, respectively. Almost 100% inhibition rate was shown at 80 µL/mL treatment for all the samples. Antioxidant activities and LDL oxidative inhibition were also measured. Gamgyul wine showed increased concentration-dependent antioxidant activities, while epigallocatechin gallate (EGCG) revealed an almost same level of activities. On the other hand, Gamgyul brandy presented little activity at any treatment concentration. Gamgyul wine in 20 µL/mL inhibited LDL oxidation approximately three times more than that of the comparative negative control, D.W. Flavanone analysis showed detections of both narirutin and hesperidin, while narirutin was the most abundant flavanone in Gamgyul wine. These overall results suggest that Gamgyul wine has the potential to be used as a functional dietary supplement and is an effective α-glucosidase inhibitor, which may be beneficial only for low to moderate intake.

***Keywords:*** *LDL oxidative inhibition, α-glucosidase inhibitor, Citrus unshiu Marc., antioxidant, Gamgyul wine, brandy*

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## 1. Introduction

Ever since the word "homo hundred," meaning the era where a long life span of 100 years becomes ubiquitous, was first used in the United Nations World Population Ageing 2009 Report, the term denoting the age of 100 years old has presently become widely known [1]. The era of homo hundred has a meaning of not just an extension of a life span for the elderly, but also poses questions toward what to prepare for one's own future that lies ahead. It largely sends a message that people in all generations must ponder and prepare together. Several topics to be sorted out in the modern times seem to be "healthy life expectancy" "health promotion," and "well-dying." Health promotion is of paramount importance since healthy life expectancy can be maintained while well-dying may be likely to occur through health promotion. Owing to a high level of economic growth and population aging presently in South Korea, adult chronic diseases, such as cardiovascular disease, diabetes mellitus and child obesity, are on the rise. Everyone knows the fact that lifestyle and dietary habits which prevent illness by implementing disease prevention measures, rather than focusing on treatment, are imperative, and difficult. Thus, chronic adult diseases may be prevented while people enjoy functional beverages. This can be done by making lifestyle habits of selecting favorable beverages, according to their functionality and by drinking them judiciously and pleasantly [2,3]. Ever since the term, "French paradox" first echoed through the broadcasting media in France, consumption of red wine increased by more than 40%. It also provided an opportunity for the establishment of many wineries outside France, such as in California, U.S.A. The "French paradox" is a term, which recapitulates a seemingly paradoxical notion that French people have a relatively low mortality rate secondary to cardiovascular disease (CVD) or less obesity, while having high consumption of red meat, as opposed to those of other European countries or the United States [4,5,6]. The fact of the matter revealed through an epidemiological study was that an active ingredient polyphenol is contained in red wine. Some French people drink wine daily, which takes credit for the favorable outcome. Researchers seemingly hit their stride in studying functional ingredients. There are various fruit wines with high functionalities in Korea, but these fruit wines are not widely enjoyed as much as grape wines. It is necessary to think about the reasons. One of the reasons is that grapes are a fruit enjoyed by many people throughout the world. A fruit that can compete with grapes is Gamgyul in Korea [7]. It was revealed long ago that Gamgyul fruit contains countless functional ingredients, such as flavonoid, hesperidin, naringin, and others [8,9]. The aim of this study is to enhance the efficiency of Jeju Gamgyul utilization through functional analysis of Gamgyul wine, which is produced from the citrus fruits of Jeju Island.

Gamgyul peels have much more functional ingredients, but are dried and utilized merely as a tea [10,11]. The consideration is that the fermentation process of alcohol extracted from complete Gamgyul fruit with their peels can produce superb functionalities of Gamgyul wine. Gamgyul and oranges are most frequently used as a juice, and their functional studies have also been conducted. Nonetheless, functional studies of Gamgyul wine have never been virtually seen, and are necessary for assessment and verification of its diverse functions. Inclusion of the fermentation process in the manufacturing procedure allows utilization of not only diverse functions of ingredients, but also large quantities of discarded peels. The production of Gamgyul wine suitable to the weather conditions in Korea can achieve "Gamgyul wine paradox." If the wine we love to drink could contribute to the effective prevention of chronic adult disease, such as cardiovascular disease, diabetes mellitus, or obesity, it would clearly be a favorable food for modern contemporaries without a doubt. However, an appropriate amount should be enjoyed more than anything. Regardless of how nutritious the food is, excessive intake of a good food is less desirable than inadequate feeding. In this study, functional differences were investigated by using two differently manufactured filtration processes, namely, diatomitic filtration (DF) and micro filtration (MF, 500 µm), which are the core technologies of quality control for Gamgyul wine production. This study investigated antioxidant, antidiabetic and LDL peroxidation inhibitory properties in Gamgyul wine produced by using two different filtration processes and distilled Gamgyul brandy for making functional analysis. It also scrutinized the types of flavanones contained in Gamgyul wine.

# 2. Materials and Methods

## 2.1. Gamgyul Wine and Chemicals

Gamgyul wine (12% v/v) and Gamgyul brandy (38% v/v) utilized in this study were provided from a wine manufacturing firm in Jeju Island, and stored in a cool and dark place. Antioxidant, antidiabetic activities and inhibition of LDL peroxidation were measured. High Performance Liquid Chromatography (HPLC) was used to analyze functional ingredients contained in Gamgyul wine at the same time. Five standard ingredients (narirutin, naringin, hesperidin, neohesperidin and hesperidin) were used for the analysis of functional ingredients. This study utilized two types of Gamgyul wines, produced independently by two filtration methods DF and MF, each of which comprises a core technology of quality control for Gamgyul wines. The stock solution of distilled liquor produced by adding several processes to Gamgyul wine and having a secondary fermentation with a final stock of 38% alcohol for 180 days to produce Gamgyul brandy. All chemicals and reagents were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MO, USA), Aldrich Chemical Co. (Steineheim, Germany), and Merck (Darmstadt, Germany).

## 2.2. Radical DPPH Scavenging Activity

The 1,1-diphenyl-2-pycryl-hydrazyl (DPPH) scavenging capacity was estimated according to a previously described procedure [12]. In brief, the reaction mixtures contained various concentrations of the extract (10, 50, 100, and 200 µg/mL) and an ethanol solution of DPPH. Freshly prepared DPPH solution was mixed into the antioxidant extract to initiate the radical-antioxidant reaction. The final concentration was 100 µM for DPPH and the total volume was 2,000µL for each reaction mixture. Incubation was performed at 37°C for 60 minutes. The absorbance at 517 nm was determined against an 80% ethanol blank. Vitamin E (10 µg/mL) was used as a positive control. Percent scavenging activity was determined by comparison with the negative control (only containing DPPH and ethanol solution) and calculated according to Eq. 1. Each experiment was performed in triplicate.

$$\text{Inhibition activity}\ (\%) = \left[\left(ABS_{control} - ABS_{sample}\right) / ABS_{control}\right]\ x100. \quad (1)$$

## 2.3. α-glucosidase Inhibition

The α-glucosidase inhibitory activities of the Gamgyul wine (13% v/v) and Gamgyul brandy (38% v/v) were determined by using a previously described procedure [13] with slight modifications. Briefly, 5 mM ρ-nitrophenyl α-glucopyranoside (ρ-NPG) as a substrate in 0.1 M phosphate buffer (pH 6.8) was used to carry out enzyme reactions. The ρ-NPG was premixed with 100 µL of 1.0 U/mL solution in 0.1 M phosphate buffer (pH 6.8). Then, samples or acarbose liquid at different concentrations (20-250 µL/mL) were added, and the mixture was further incubated at 37°C for 30 minutes. Further reactions were terminated by adding 0.9 mL of 0.1M Na2CO3 solution. The absorbance of the released ρ-NPG was measured at 405 nm, and the inhibitory activity was calculated according to Eq. 2. Each experiment was performed in triplicate.

$$\text{Inhibitory activity}\ (\%) = \left[\left(ABS_{control} - ABS_{sample}\right) / ABS_{control}\right]\ x100. \quad (2)$$

## 2.4. LDL Oxidation

A human LDL solution was purchased from the Sigma Company. Then, ethylenediaminetetraacetic acid (EDTA) in the LDL solution was removed by making two passages through the Econo-Pac 10G desalting column (Bio-Rad, Richmond, CA, USA), hydrated, and eluted with a phosphate-buffered saline (PBS) solution. Protein concentration was determined with the Bradford reagent (Bio-Rad, City, State, Country), using bovine serum albumin as a reference. For LDL oxidation, 50 μg/ml of LDL was treated with 5.0 μM $CuSO_4$ with PBS to make a final volume of 1.25 ml with or without extracts of Gamgyul wine, which was then evaporated and dissolved with distilled water. Finally, the treated Gamgyul wine was incubated at 37°C for 24 hours. The oxidized LDL was measured by using a commercially available kit (Bioxytech MDA-586TM, OxisResearch, City, State, U.S.A.). The MDA-586 method is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (NMPI), with malondialdehyde (MDA) at 45°C. One molecule of MDA reacts with two molecules of NMPI to form a stable carbocyanine dye, which was intensely colored with a maximum absorption at 586 nm.

## 2.5. Determination of Gamgyul Wine Flavanones

HPLC was performed using a Shimadzu model LC-2010 CHT system equipped with a vacuum degasser, a quaternary pump, an Autosampler, a thermostatted column compartment and a UV-visible spectra detector. In an effort to analyze the types and concentrations of flavanones contained in Gamgyul wine, this study utilized 5 standard ingredients, namely, narirutin, naringin, hesperidin, neohesperidin, and hesperitin. Gamgyul wine was diluted with a solution of 10 % Methanol: DMSO (1:1). Then, the diluted mixture was filtered through the 0.45 μm cellulose acetate filter before carrying out HPLC analysis.

## 2.6. Statistical Analysis

The samples were assayed at least three times in each test, and the results were expressed as the mean±standard deviation (SD) before the data was analyzed. Percent maximum inhibition values were analyzed by unpaired t-tests. Differences between means were considered significant if $p<0.05$, as denoted in appilicable figures, and the text.

# 3. Results

**DPPH Radical Scavenging Activity**: The DPPH radical scavenging activities of varying concentrations for 2 Gamgyul wines and 1 brandy are shown in Figure 1.

In both wines with different filtration processes, such as diatomite filtration (DF) and microfiltration (MF), significant dose-dependent DPPH radical scavenging capacities were detected. At the concentration of 400 μL/mL, both types of Gamgyul wines showed nearly 90% or more DPPH radical scavenging activities. However, Gamgyul brandy unexpectedly showed low activities regardless of processing concentrations. They were compared with epigallocatechin gallate (EGCG), known widely as antioxidant substance at the 400 μL/mL concentration, at which the rate of free radial removal would be the highest (Figure 2).

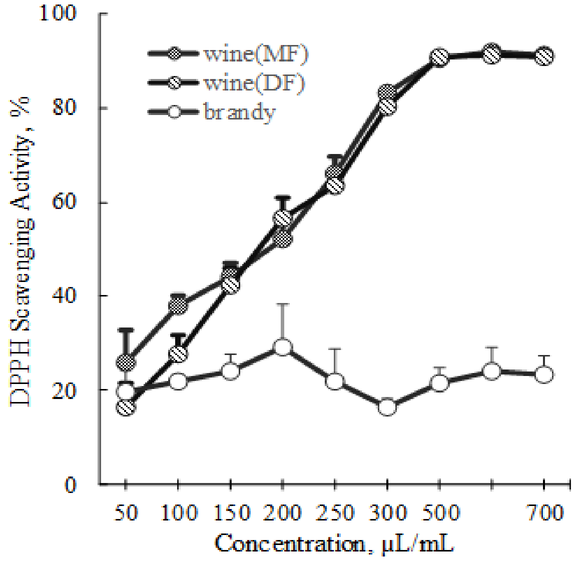


**Figure 1.** Dose-dependent DPPH radical scavenging activities of Gamgyul wines and brandy are revealed. The values are shown as the mean±standard deviation (n=3)

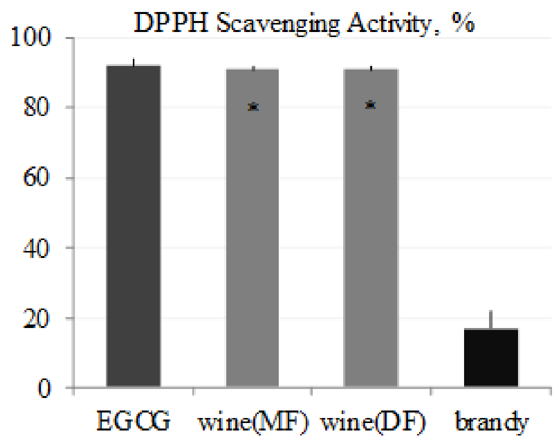


**Figure 2.** The effect of DPPH scavenging activities of Gamgyul wines and brandy at the 400μL/mL concentration, and that of EGCG at 50μM.. The values are mean ±standard deviation (±SD). *$p<0.05$ compared with EGCG

**α-glucosidase Inhibition:** The inhibitory effect of diatomite filtered (DF) Gamgyul wine was nearly the identical to that of EGCG. However, the other microfiltered (MF) Gamgyul wine showed little inhibitory activities at very low concentrations, such as 20 μL/mL (Figure 3). Gamgyul brandy showed rather interesting results. Antioxidation activities were largely not present in Gamgyul brandy, and naturally no antidiabetic effect was anticipated. On the contrary, Gamgyul brandy showed a 50% inhibitory effect even at 20 μL/ml in comparison to that of two Gamgyul wines. The enzymatic activities of α-glucosidase inhibition were nearly 100% at 60 μL/ml. This is the result of more superb activities than that of the already-known EGCG.

**LDL Oxidation:** Recently, numerous studies were reported that the oxidation of blood LDL plays an important role in coronary diseases or atherosclerosis. Thus, the significance of inhibitory effect on LDL oxidation has been emphasized. Antioxidants have been reported to have inhibitory activities against LDL oxidation. Especially, berries are reported to have exceptional antioxidant activities as well. The results of this study revealed very high DPPH activities of free radical scavenging for two Gamgyul wines, except for Gamgyul brandy. Thus, an exceptional inhibitory effect of LDL oxidation was expected. Accordingly, diatomite filtrated (DF) wine was utilized for the assessment of nhibitory capacity of LDL oxidation as indirect measurements of MDA generation. EGCG and distilled water (DW) were used as positive and negative control, respectively (Figure 4).

The inhibitory effect of Gamgyul wine was not as high as that of EGCG-the positive control. Nevertheless, its inhibitory capacity of LDL oxidation at the processing concentration of 20 μL/mL was approximately 3 times that of the negative control, DW.

**Determination of Gamgyul Wine Flavanones:** HPLC was used to examine the types and concentrations of the functional ingredients contained in two Gamgyul wines, each of which was extracted by using a different filtration method. This investigation utilized five standard ingredients narirutin, naringin, hesperidin, neohesperidin, and hesperetin (Figure 5).

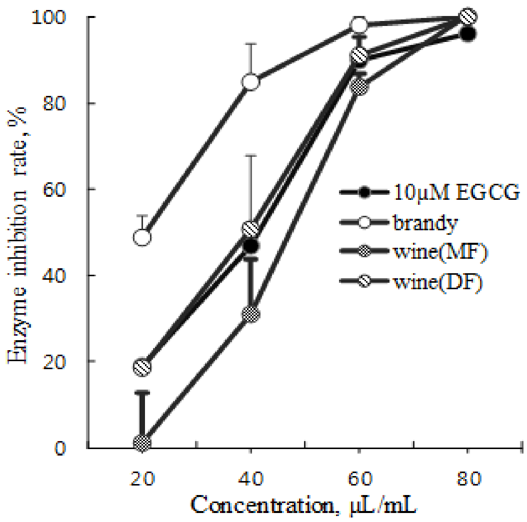


**Figure 3.** Activities of α-glucosidase inhibition for two Gamgyul wines and one brandy. EGCG was used as a positive control. The values are mean ±standard deviation (±SD)

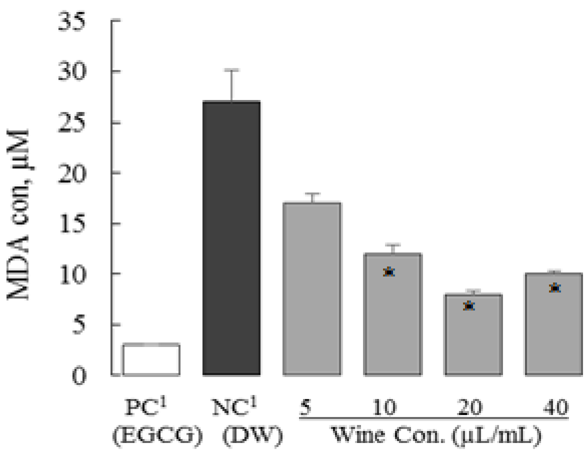


**Figure 4.** It shows the effect of Gamgyul wine, which is the end product of evaporated alcohol, treated with distilled water, and processed with Cu2+-induced lipoprotein oxidation. The results are expressed as the mean±standard deviation (n=3). PC[1] and NC[1] denote positive and negative control, respectively, and EGCG was used as a PC. *p<0.05 compared with NC, DW

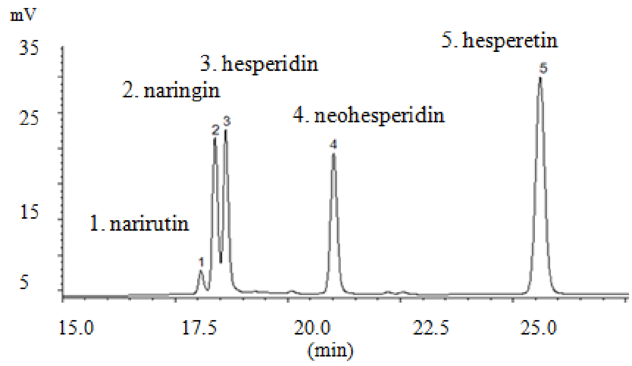


**Figure 5.** Reverse-phase HPLC chromatogram of a standard mixture of the flavanones

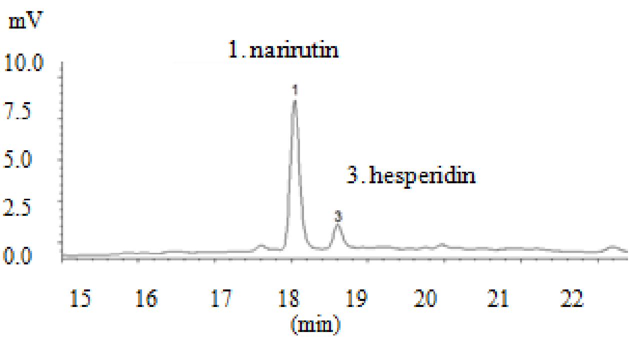


**Figure 6.** Reverse-phase HPLC chromatogram of a 10% diluted Gamgyul wine

The results of the analysis attained from diluting the DF Gamgyul wine stock by 10% showed that HPLC could detect narirutin and hesperidin at retention times of 18.039 and 18.601 minutes, respectively (Figure 6). As shown in the HPLC chromatogram, the concentrations of narirutin and hesperidin were 1415.42 ppm and 28.25 ppm, respectively. The level of narirutin was 50 times that of hesperidin, indicating that the ingredient of Gamgyul wine has largely consisted of narirutin. The narirutin concentration of MF Gamgyul wine was approximately 30 ppm higher than that of DF Gamgyul wine, but the concentration of hesperidin was nearly identical. Thus, there was no significant difference in concentration of these ingredients between the two different filtration methods.

## 4. Discussion

Citrus is one of the most enjoyed fruit in the world, and has been recognized as a functional food. However, citrus fruits are consumed as fresh fruits or processed as juice by extraction. Most studies of functionalities have been carried out for citrus extracts and processed juice [7,14,15]. Furthermore, functional studies of wines are largely related to red wines. Notwithstanding superb functions possessed by Gamgyul fruits, there have been nearly no research on Gamgyul wine. This study utilized Gamgyul wine, produced by using whole fruits of fruit pulp and peels, as well as Gamgyul brandy produced through distillation of Gamgyul wine and a secondary fermentation process. It also scrutinized antioxidant and antidiabetic properties, as well as the attributes of LDL oxidation inhibition. Along with analysis of the types of flavanones content in Gamgyul wine, this study puts effort to assess functionalities of Gamgyul wines.

In this study, both Gamgyul wines and brandy showed exceptional antidiabetic effect. In particular, Gamgyul brandy showed α-glucosidase inhibition activities of approximately 2.5 times that of Gamgyul wine at the low concentration of 20 µL/ml. The extent of its activation is 2.5 times higher than that of EGCG-the functional ingredients of green tea. The α-glucosidase inhibition activity of EGCG was shown to be superior to that of acarbose drug, which is available as postprandial blood glucose inhibitors [16,17,18]. The acarbose drug is used as an α-glucosidase inhibitor (AGI) to inhibit starch digestion in the small intestine. In particular, the assumption is that controlling postprandial hyperglycemia is an efficient therapeutic approach in the management of type 2 diabetes mellitus. This process is generally realized by retarding key enzyme α-glucosidase in the digestive system linked to the absorption of glucose. The data from this study suggests that Gamgyul wine and brandy show promise as potential α-glucosidase inhibitors that inhibit digestion of dietary starches without side effects. Already in 2006, Sugihara et. al. [19] found that citrus might act as a suppressor against type 2 diabetes. They investigated the effects of chronic administration of a fruit extract (*Citrus unshiu* Marc.) on glucose tolerance in GK rats, a model of type 2 diabetes. Recent studies also suggested that citrus pectin could ameliorate type 2 diabetes and potentially be used as an adjuvant treatment [20]. It is a well-known fact that aside from citrus pulp, the peels are good sources of bioactive compound and minerals [11]. However, the effect of Gamgyul brandy is higher than that of Gamgyul wine. Further study is needed to find out whether such a higher effect of Gamgyul brandy is due to a simple concentrated effect attained by the evaporation process of Gamgyul wine, or due to heightened reactions following secondary fermentation of Gamgyul wine for 120 days. Besides having a significant AGI effect, Gamgyul wine, not the brandy, showed very high antioxidant activities which proved to be effective LDL oxidation inhibitors in this study. This may be linked to phenolic compounds. That is, phenolic compounds were reported to be effective α-glucosidase inhibitors [21,22,23]. Unlike Gamgyul brandy, Gamgyul wine shows high antioxidant activities and LDL oxidation inhibition. Because oxidative stress is considered to play a vital role in chronic inflammatory disease like diabetes mellitus and its complications, namely, a proper balance between oxidative stress and antioxidants is necessary [24]. It is quite a well-known fact that citrus flavanones like naringenin and hesperetin, aglycones of naringin and hesperidin, reduce pathogenesis of diabetes mellitus and its related complications. DPPH radical scavenging activities and total phenolic contents were measured by using different extraction methods, such as hot water extraction and 70% ethanol extraction. The results showed that ethanol extract was remarkably better than hot water extract. It was reported that naringin, known as an antioxidant flavonoid, was measured only from 70% ethanol extracts [25]. Studies done by Sun et al. [10] on citrus fruits, pomelo, also showed that only naringin and tangeretin were present. Whereas, grapefruit compounds studied by Robards et al. [9] showed that they had hesperidin and narirutin. These are exactly the same compounds found in this study on Gamgyul wine. The study results on flavanones, the major flavonoids found in the citrus fruits, showed that nariruatin was present in much higher quantity than hesperidin, almost 50 times more. The study also indicated that the main flavanone of Gamgyul wine was narirutin Moura et al. [26] showed that hesperidin was the main flavanone in orange juice, but the narirutin was found in market tangor. The narirutin fraction from the peels of *Citrus unshiu* was investigated and found that the fruits had a potential to be used as a functional dietary supplement and an effective anti-inflammatory agent. The fruits also decreased alcoholic liver disease in mice [24]. In consideration of the results of this study and those of other research reports, the types and concentrations of flavanones are largely affected by extraction methods and their variety. In accordance with reports of studies using only fruit pulp and peels, flavanone contents were remarkably high in fruit peels. In fact, it was reported that all of the individual flavanones and carotenoids significantly increased throughout the fermentation or enzymatic treatment in orange juice [27]. These study results support our results of Gamgyul wine and Gamyul brandy with the process of the whole fruits. Furthermore, alcoholic fermentation may support health promotion, increasing its validity as food products. Finally, there was no significant difference in efficacy between these two types of filtrating processes of diatomite fitration and micro filtration.

## 5. Conclusion

If the wine we love to drink could contribute to the effective prevention of chronic adult disease, such as cardiovascular disease, diabetes mellitus, or obesity, it would clearly be a favorable food for modern contemporaries without a doubt. However, an appropriate amount should be enjoyed more than anything. Regardless of how nutritious the food is, excessive intake of a good food is less desirable than inadequate feeding.

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