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Radical Scavenging Activities of Korean Traditional Rice Wine, *Takju*

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Abstract

The principal objective of this study was to assess the radical scavenging activities and total polyphenols, flavonoids, and flavonol contents of *takju*, a Korean traditional rice wine. The antioxidant properties of the wine and *takju* samples were evaluated using five distinct assays: specifically, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); hydroxyl; superoxide anion; and nitric oxide (NO) radical-scavenging activity assays. In this study, the *takju* evidenced strong scavenging activities against the hydroxyl, superoxide anion, and NO radicals. Furthermore, the total polyphenol contents of T-2 were similar to those previously observed in white wine ($p<0.05$). However, the flavonoids and flavonol contents of all *takju* samples were significantly ($p<0.05$) lower than that of white wine. The results of this study show that *takju* possesses a powerful radical scavenging activity against a variety of oxidative systems. The findings of this study also indicate that *takju* should be considered a useful antioxidant, and that their functional compound reduces oxidative stress.

Key words: *takju*, radical scavenging activity, total polyphenol, flavonoids, flavonol

INTRODUCTION

Since the beginning of human civilization, an intimate companionship has existed between the human being, his fare, and the fermentative activities of microorganisms. These fermentative activities have long been exploited in the production of fermented foods and beverages (1). Several types of cereal-based fermented drinks are produced around the world, and these can be classified on the basis of the raw materials utilized or the type of fermentation involved in the manufacturing process. Alcoholic fermented beverages can be classified into wines and beers, whereas the vast majority of non-alcoholic fermentations are souring, primarily lactic acid-based fermentations (2,3).

Drinking patterns and type of alcohol consumed may be relevant to the incidence of coronary heart disease in men (4), and it has been previously suggested that components other than the alcohol in the beverages, such as flavonoids, might also be implicated in observed protective effects of these beverages against coronary heart disease. Oxidative stress is the initial step in the pathogenesis of atherosclerotic disease. There is now mounting evidence from animal models and observational studies suggesting that the consumption of certain foods can reduce oxidative stress (5).

This greater protection may be attributable to the polyphenol contents of wines, which are particularly abundant in wine, because poyphenols function as reactive oxygen species (ROS) scavengers and metal-chelators. Numerous papers have been published on wines, and the antioxidant properties of wines have been extensively correlated with their polyphenol contents (6-9).

Korean rice wine, or *takju*, is an alcoholic beverage brewed with the yeast *Saccharomyces cerevisiae*. *Takju* is similar to grape wine, except that it is made from rice. The consumption of wine containing phenolic compounds has been previously reported by many researchers to increase the antioxidant content and status of human blood plasma (10,11). Few studies have yet been conducted, however, to identify the novel functional compounds in *takju*. As a consequence of these studies, though, a variety of antioxidant properties have been attributed to *takju*, and this has generated world-

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wide attention. Indeed, the active substances that have been isolated from *takju* include total polyphenols, flavonoids, and flavonols (12,13), which evidence a wide range of chemical characteristics and manifest a variety of antioxidant activities.

Although *takju* is well known to exhibit various biological activities, there has been a dearth of studies thus far conducted to evaluate the effects of *takju* on health. Therefore, we investigated the antioxidant compounds contained in *takju*, and evaluated the ability of these compounds to scavenge the following free radical types: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl, superoxide anion, and nitric oxide (NO). This study may prove useful to researchers attempting to improve the quality of *takju*, or who are addressing the functional compounds contained in *takju*.

MATERIALS AND METHODS

Chemicals and materials

Folin-Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), p-dimethylamino cinnamaldehyde (DMACA), phenazine methosulfate (PMS), and deoxyribose were purchased from Sigma-Aldrich (St. Louis, USA). 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Fluka (Steinheim, Germany). All other reagents used were of analytical grade.

Five *takju* samples were purchased from a local liquor shop in Seoul, Korea, and stored at 4°C until use. The *takju* samples were centrifuged for 20 min at 8,000 rpm, and the supernatants were used for subsequent analyses.

DPPH radical scavenging activity

The DPPH radical scavenging activity was assessed via the method previously described by Cheung et al. (14), with some modifications. In brief, 0.4 mL of 0.2 mM DPPH ethanolic solution was mixed with 0.1 mL of sample. The mixture was then shaken vigorously and allowed to stand for 10 min under subdued light, after which the absorbance was measured at 520 nm.

ABTS radical scavenging activity

The ABTS radical cation scavenging activity was measured in accordance with the method described by Re et al. (15), with some modifications. The ABTS radical cation was generated via the addition of 7 mM ABTS to a 2.45 mM potassium persulfate solution, after which the mixture was allowed to stand overnight in darkness at room temperature. The ABTS radical cation solution was then diluted with distilled water to achieve an absorbance of 1.4～1.5 at 414 nm (molar extinction coefficient, $\varepsilon=3.6\times10^4$ M^{-1} cm^{-1}) (16). Next, 1 mL of diluted ABTS radical cation solution was added to a 50 μL sample. Finally, the absorbance was measured at 414 nm.

Hydroxyl radical scavenging activity

In order to assess the hydroxyl free radical scavenging activity of wines, the deoxyribose method was used, as described by Halliwell et al. (17), with some slight modifications. Approximately 0.4 mL of 20 mM phosphate buffer (pH 7.4), 0.1 mL of 60 mM deoxyribose, 0.1 mL of 10 mM hydrogen peroxide, 0.1 mL of 1mM ferric chloride, 0.1 mL of 1.04 mM EDTA, 0.1 mL samples, and the final 0.1 mL of 2 mM ascorbic acid were mixed. The reaction mixtures were incubated for 1 hr at 37°C, after which 1 mL of 17 mM TBA and 1 mL of 17 mM TCA were added. The mixture was then boiled for 15 min, ice-cooled, and measured for absorbance at 532 nm. Distilled water in lieu of extract was utilized as a blank and the sample solution without added deoxyribose was used as a sample blank.

Superoxide anion radical scavenging activity

Superoxide anion scavenging activity was measured based on the method described by Robak and Gryglewski (18). Superoxide radicals were generated in a PMS-NADH system via the oxidation of NADH and then assayed by the reduction of NBT. The superoxide radicals were generated in 0.3 mL of sodium phosphate buffer (100 mM, pH 7.4) containing 0.3 mL of NBT (150 μM) solution, 0.3 mL of NADH (468 μM) solution in sodium phosphate buffer, and 0.3 mL different samples in mixture. 0.3 mL of PMS solution (60 μM) was then added to the mixture. The reaction mixture was incubated for 5 min at 25°C and the absorbance was measured at 560 nm.

NO radical scavenging activity

The scavenging effect of NO was measured in accordance with the method of Marcocci et al. (19). In brief, sodium nitroprussiate dehydrate (5 mM) in phosphate-buffered saline (PBS) (pH 7.4) at a dose of 30 μL was mixed with 90 μL of different test samples, then incubated for 150 min at 25°C. After incubation, the nitrite generated from sodium nitroprussiate dehydrate was measured using Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% 1-naphthlethylene diamine dihydrochloride in water). The absorbance was then read immediately at 570 nm.

Total polyphenol, flavanoids and flavonols contents

The total polyphenol contents were determined via the Folin-Ciocalteu method (20) adapted to a micro scale.

In brief, 0.79 mL of distilled water, 0.01 mL of appropriately diluted sample, and 0.05 mL of phenol reagent were added to a 1.5 mL Eppendorf tube, then mixed. After exactly 1 min, 0.15 mL of 20% sodium carbonate was added, and the mixture was mixed and allowed to stand at room temperature for 120 min. The absorbance was then read at 750 nm and the total polyphenol content was calculated from a calibration curve, using gallic acid as a standard.

The flavonoid contents were determined via the Folin-Denis method (21) adapted to a micro scale. In brief, 0.25 mL of samples and 0.075 mL of sodium nitrite were mixed. After exactly 5 min, 0.15 mL of 10% alumimum chloride was added, and after exactly 6 min 0.5 mL of 1 M sodium hydroxide, 0.275 mL of distilled water was then added. Afterward, the absorbance at 510 nm and the flavonoid contents were calculated from a calibration curve, using catechin as a standard.

The flavonol contents were estimated via the DMACA method, as described by Li et al. (22). In brief, 0.2 mL of sample diluted to 1:100 with MeOH was added to a 1.5 mL Eppendorf tube containing 1 mL of 0.1% DMACA solution in 1 N HCl in MeOH. The mixture was then vortexed and allowed to react for 10 min at room temperature. Afterward, the absorbance at 640 nm was read against a blank that had been prepared in accordance with the same method, without the DMACA. The flavonol content was then estimated from a calibration curve constructed via the plotting of known catechin solutions.

Statistical analysis

All values shown are the means of triplicate determinations. All statistical analyses were conducted using the Statistical Package for Social Sciences, version 12.0 (SPSS Inc., USA). The differences among samples were statistically evaluated via one-way analysis of variance (ANOVA) and Duncan's multiple tests. All data were evaluated at the 5% significance level using two-sided tests, and are expressed as the means±standard deviations (SD).

RESULTS AND DISCUSSION

Radical scavenging activities

In this study, the antioxidant properties of wine and *takju* samples were evaluated via five distinct assays: specifically, DPPH; ABTS; hydroxyl; superoxide anion; and NO radical scavenging activity assays. These methods differ in terms of their assay principles and experimental conditions. As multiple reaction characteristics and mechanisms are usually involved, no single assay can reflect accurately all antioxidants within a mixed or complex system. Thus, in order to generate a full antioxidant capacity profile, different antioxidant capacity assays may be required.

DPPH is a free radical compound which has been extensively used to determine the free radical-scavenging ability of various samples (23). This method is based on the reduction of DPPH in alcoholic solution in the presence of a hydrogen-donating antioxidant, owing to the formation of the non-radical form DPPH-H in the reaction. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule, and DPPH levels are significantly reduced upon exposure to proton radical scavengers (24). The DPPH radical-scavenging activities of wine and *takju* are shown in Fig. 1. White wine evidenced intense DPPH radical scavenging. The DPPH radical scavenging activity of white wine (93.18%) was significantly ($p<0.05$) higher than that of all *takju* samples. Overall, T-5 evidenced the highest levels of DPPH radical scavenging activity among the *takju* samples, which occurred in the following order: T-5 (27.00%)> T-1 (19.86%)> T-4 (19.29%)> T-2 (17.12%)> T-3 (7.99%).

The ABTS and DPPH radicals are the two most extensively used and stable chromogen compounds for measurements of the antioxidant activity of biological material (25). The results of the ABTS assay were similar to those of the DPPH assay (Fig. 2) but the *takju* samples evidenced higher ABTS scavenging activity than was observed for the DPPH radicals. The white wine (96.46%) also evidenced a radical activity significantly higher ($p<0.05$) than those of all the *takju*

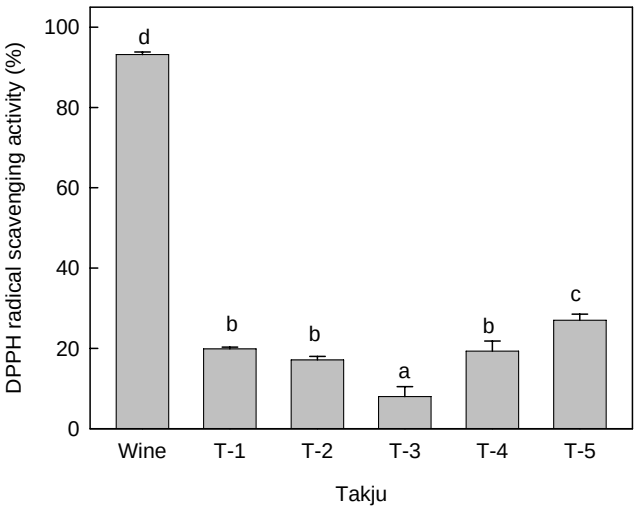

Fig. 1. DPPH radical scavenging activities of various *takju*s. Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Bars are the mean±SD of triplicate determinations.

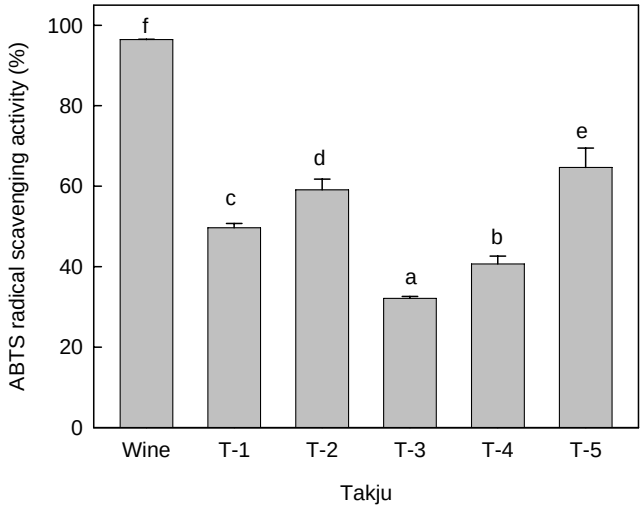

Fig. 2. ABTS radical scavenging activities of various *takju*s. Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Bars are the mean±SD of triplicate determinations.

samples. Specifically, the ABTS assay showed that the relative antioxidant activities of the *takju* occurred in the following order: T-5 (61.99%)> T-2 (59.09%)> T-1 (49.68%)> T-4 (40.67%)> T-3 (32.08%).

The hydroxyl radical scavenging activities of wine and *takju* are shown in Fig. 3. The hydroxyl radical is a very reactive ROS with a short half-life, and is considered to be responsible for much of the biological damage inherent to free radical pathology (26). This radical has the ability to cause strand breakage in DNA, which is a contributing factor to carcinogenesis, mutagenesis, and cytotoxicity. Moreover, hydroxyl radicals have been identified as one of the rapid initiators of the lipid peroxidation process, via the abstraction of hydrogen atoms from unsaturated fatty acids (27). In this study, the hydroxyl radical scavenging activities of wine and *takju* were not shown to be significantly different. Wine, T-1, T-2, T-3, T-4, and T-5 exhibited hydroxyl radical scavenging activities of 83.48%, 88.28%, 83.29%, 84.97%, 83.33%, and 84.77%, respectively.

The superoxide anion radical scavenging activities of wine and *takju* are shown in Fig. 4. Over-production of superoxide anion radical has long been known as the starting point of ROS/RNS accumulation in cells, contributing to redox imbalance and other associated deleterious physiological consequences (28). One simple, rapid, and low-cost method for the evaluation of the superoxide anion scavenging activity of extracts is based on the triad NADH, reduced PMS dioxygen as a source of the superoxide anion radical. The reduction of NBT, as a superoxide anion scavenging compound, results in a stable formazan. Perhaps the antioxidant activity observed in passion fruit, for example, is not only attributable to its contained phenolic compounds, but also to important contributions from other superoxide anion radical scavengers, including essential oils, carotenoids, and vitamins (29). The activity of *takju* samples to scavenge superoxide anion radicals was significantly ($p<0.05$) lower than that of white wine (83.18%), except for T-3. However, T-4 manifested the lowest levels of superoxide anion radical scavenging activity among the *takju* samples. Our results show that both wine and *takju* evidence superoxide radical scavenging activity, which were, in decreasing order: T-3 (87.55%)> T-1 (83.70%) > T-5 (82.64%)> T-2 (80.75%)> T-4 (21.05%).

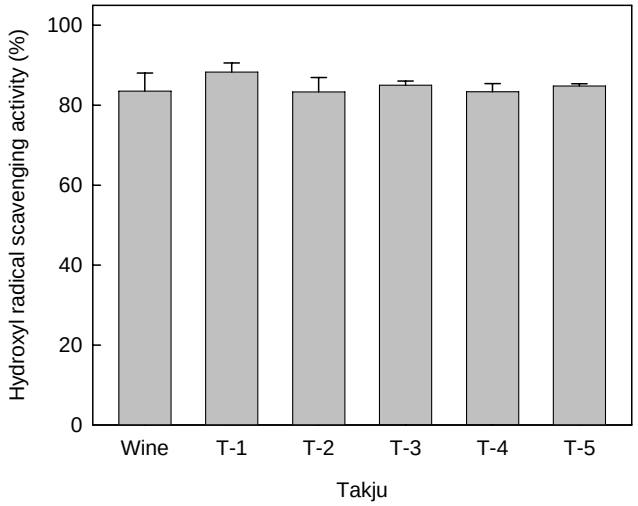

Fig. 3. Hydroxyl radical scavenging activities of various *takju*s. Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Bars are the mean±SD of triplicate determinations.

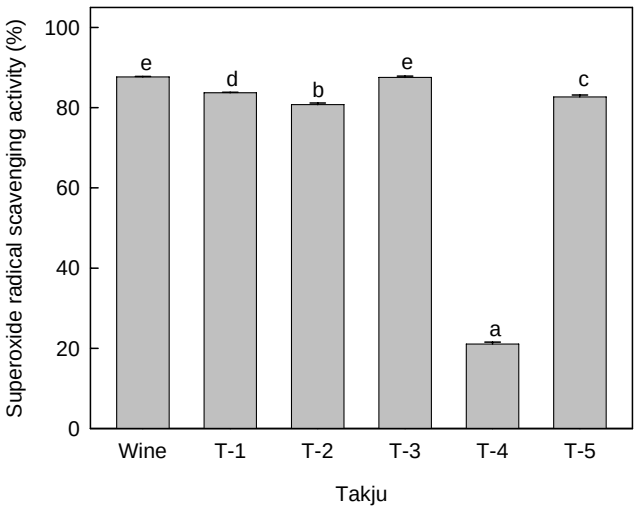

Fig. 4. Superoxide radical scavenging activities of various *takju*s. Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Bars are the mean±SD of triplicate determinations.

The NO radical scavenging activities of wine and *takju* are shown in Fig. 5. NO is a potentially toxic agent with a free radical character, and is therefore responsible for a variety of physiologic and pathologic events (19). The activity of white wine to scavenge NO radicals was significantly ($p<0.05$) higher than all *takju* samples (white wine (83.89%)) We noted moderate NO scavenging activity in the *takju* samples (in decreasing order): T-3 (77.75%)> T-1 (67.13%)> T-5 (56.80%)> T-2 (50.00%)> T-4 (44.32%), respectively.

The radical scavenging activities of the hydroxyl, superoxide anion, and NO radical by *takju* were shown to be substantially more profound than their ability to scavenge the DPPH and ABTS radicals. Wang et al. (30) determined that some compounds with ABTS scavenging activity did not evidence DPPH scavenging activity. This further illustrates the activity of *takju* to scavenge different free radicals in different systems, thereby indicating that the constituents of *takju* may prove to be useful therapeutic agents for the treatment of radical related pathological damage. In this study, the *takju* evidenced profound scavenging activities against the hydroxyl, superoxide anion, and NO radicals. The differences in the raw materials and *nuruk* contribute to differences in the compositions of *takju*. Furthermore, the fermentation techniques and culture conditions employed also influence the composition of *takju*, owing to their influence on the biochemical synthesis of a variety of compounds. Accordingly, the results of many studies have shown that the contents of chemical compositions, including total sugars (31), pH (13), ethanol (32), total acid (31,32), amino-nitrogen (33), and reducing sugar (34) contents vary among types of *takju*.

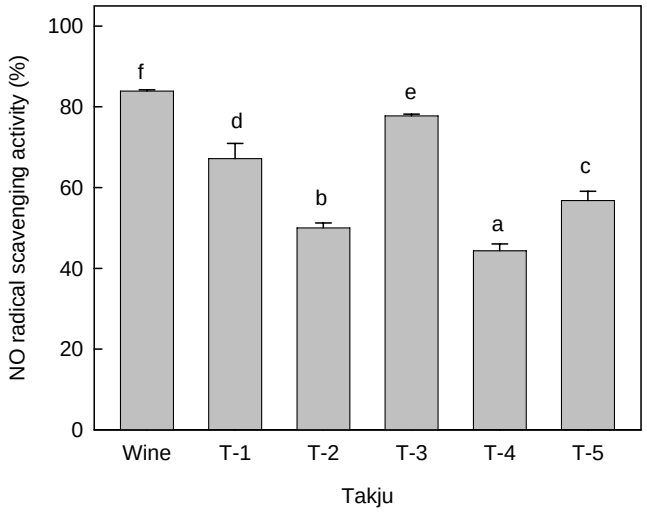

Fig. 5. NO radical scavenging activities of various *takju*s. Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Bars are the mean±SD of triplicate determinations.

Free radicals have been implicated in the pathogenesis of many diseases, including myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, and cancer-initiation, as well as in the aging process (35). There is a considerable amount of evidence now to suggest that antioxidants might help in preventing these diseases, as they have the capacity to quench free radicals (36). Although some synthetic antioxidants, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), evidence potent free radical scavenging effects, they have also been demonstrated to exert toxicological effects as compared with natural antioxidants (37). Thus, the demand for alternative and safe antioxidants derived from natural sources has grown gradually.

Recently, natural foods and food-derived antioxidants, such as vitamins and phenolic phytochemicals, have become the focus of increased attention, as they are known to function as chemopreventive agents against oxidative damage. Additionally, many studies have been conducted thus far in an attempt to elucidate the potential health benefits of dietary phenolic phytochemicals with strong antioxidant activities. For example, wine is currently receiving a great deal of attention as a source of dietary antioxidants, owing to the possible link between a moderate intake of wine and the low incidence of coronary heart disease in the south of France (38,39). Wine harbors phenolic compounds with profound *in vitro* free radical scavenging activity as compared to other alcoholic beverages (40). Although ethanol exerts a positive effect on lowering blood cholesterol levels, it has been proposed that the phenolic components are involved in the alleviation of coronary heart disease (41). Furthermore, the intake of wine containing phenolic compounds reportedly results in an increase in the antioxidant content and status of human blood plasma (10,11). *Takju* is an alcoholic beverage brewed by fermentation; *takju* is similar to grape wine, except in that it is produced from rice. In a study of the antioxidant activity of *takju*, Kim et al. (42) reported that the electron donating activity and superoxide dismutase (SOD)-like activity of *takju* (*Ganoderma lucidum*) were 16.5% and 42.2%, respectively.

Total polyphenols, flavanoids and flavonols contents

The total polyphenol, flavonoid, and flavonol contents were measured for white wine and *takju*. The results are provided in Table 1. The total polyphenol contents of wine (77.89 μg/mL) and T-2 (76.13 μg/mL) were significantly ($p<0.05$) higher than those of T-5 (58.94 μg/mL), T-1 (52.98 μg/mL), T-3 (24.91 μg/mL) and T-4

Table 1. Total polyphenol, flavonoid, and flavonol contents of *takju*s

Sample	Total polyphenols (μg/mL)	Flavonoids (μg/mL)	Flavonols (μg/mL)
Wine	77.89±2.78[d]	37.72±0.53[d]	3.55±0.24[c]
T-1	52.98±2.65[b]	10.04±0.70[b]	0.66±0.01[a]
T-2	76.13±1.61[d]	9.73±0.27[b]	1.01±0.01[ab]
T-3	24.91±3.99[a]	4.96±0.27[a]	1.08±0.08[ab]
T-4	21.40±1.61[a]	9.27±0.70[b]	1.37±0.41[b]
T-5	58.94±1.05[c]	11.88±0.53[d]	0.95±0.04[d]

Different letters indicate significant differences ($p<0.05$) among samples as determined by Duncan's multiple range test. Values shown are the mean±SD of triplicate determinations.

(21.40 μg/mL) whereas the total polyphenol contents were (in decreasing order): T-2> T-5> T-1> T-3> T-4.

Polyphenols are the principal plant compounds evidencing antioxidant activity. This activity is believed to be primarily due to their redox properties (43), which perform important functions in the adsorption and neutralization of free radicals, the quenching of singlet and triplet oxygen, and the decomposition of peroxides.

However, the flavonoids and flavonol contents of all *takju* samples were significantly ($p<0.05$) lower than those of white wine. Many studies have been conducted in attempts to identify novel functional compounds in *takju*. Since the functionality in *takju*, contributed by such compounds as the chitooligosaccharides from *koji* mold, was discovered, many new functional variants of traditional rice wines have been developed. Concurrent with this development, the demand for *takju* has increased considerably (42). As a result, various bioactive properties of *takju* have been identified, and this has generated worldwide attention. Indeed, the various active substances thus far isolated from *takju* include polyphenols, polysaccharides, and polysaccharide-peptide complexes (12,13), which exhibit a wide range of chemical characteristics and exert a variety of biological effects, including antioxidant activity and immuno-modulating activity.

CONCLUSION

The results of this study clearly demonstrate that *takju* possesses an effective scavenging activity against free radicals including DPPH, ABTS, hydroxyl, superoxide anion, and NO radicals, and also contain polyphenols, flavonoids, and flavonol. The findings of this study indicate that *takju* manifests powerful radical scavenging activity against various oxidative systems. Further, our results indicate that *takju* may prove to be a useful antioxidant, and that its functional compounds reduce oxidative stress. To date, no studies have yet been conducted comparing the biological activities of *takju* and wine, which is also known to contain phenolic compounds with significant free radical scavenging activity. This study may prove useful for researchers who seek to improve the quality of *takju*, or who are addressing the functional compounds in *takju*.

Additionally, the total antioxidant activity of wines was well-correlated with their phenolic contents and compositions, whereas different phenolic groups were not shown to contribute equally to the total antioxidant activity. *Takju* should be regarded as a potential nutraceutic resource, capable of constituting a significant low cost, nutritional dietary beverage for worldwide customers. On the basis of the data reported herein, additional studies should be conducted to characterize the bioactive compounds responsible for these observed properties of *takju*.

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